AD_____

Award Number: DAMD17-96-1-6287

TITLE: Gene Therapy Mediated Breast Cancer Immunity

PRINCIPAL INVESTIGATOR: NESBIT, HEIKE K.

CONTRACTING ORGANIZATION: University of Pennsylvania
                          Philadelphia, Pennsylvania   15104

REPORT DATE: September 1999

TYPE OF REPORT: **Final**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
               Fort Detrick, Maryland   21702-5012

DISTRIBUTION STATEMENT: Authorized for Release;
                        Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20001013 105

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 1999	3. REPORT TYPE AND DATES COVERED Final (15 Aug 96 – 14 Aug 99)	

4. TITLE AND SUBTITLE
Gene Therapy Mediated Breast Cancer Immunity

5. FUNDING NUMBERS
DAMD17-96-1-6287

6. AUTHOR(S)
NESBIT, HEIKE K.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Pennsylvania
Philadelphia, Pennsylvania 15104

E-MAIL:
nesbit@mail.med.upenn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Authorized for Release;
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The goal of the study was to assess the efficiency of B7-1 expressing breast cancer cells as a tumor vaccine. We showed that adenovirally delivered B7-1 expression on mammary carcinoma cells did not result in tumor rejection in vivo and failed to activate allogeneic T cells in vitro. We provided evidence that the lack of T cell stimulation by B7-1 expressing breast cancer cells was due to secretion of PGE_2. PGE_2 is produced by the cyclooxygenase (COX) mediated oxidation of arachidonic acid. The inhibition of COX activity in B7-1 expressing MDA-MB 231 cells restored the proliferative response of T cells. To further support the finding that PGE_2 inhibits B7-1 induced T cell responses we created B7-1 positive, COX-1 expressing HBL-100 breast epithelial cells and WM9 melanoma cells. T cells were stimulated to proliferate by B7-1 expressing HBL-100 cells or WM9 cells, whereas T cell proliferation was inhibited when both B7-1 and COX-1 were expressed. Therefore, PGE_2 may limit the use of breast cancer vaccines based on B7-1 expressing breast cancer cells.

14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 68
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Heike Nesbit   8/27/99
PI - Signature   Date

Table of contents

Introduction

B7-1 expression on tumor cells leads to tumor rejection and anti-tumor immunity (1) and forms the basis of several clinical immunotherapy trials. However, B7-1 expression is not as effective in providing protection against poorly immunogenic tumors, such as breast cancer. It is not yet known why B7-1 therapy cannot be applied to certain tumor systems.

One hypothesis is that through the production of immunosuppressive factors breast cancer cells evade destruction by the immune system. We have shown that breast cancer cells produce high amounts of immunosuppressive PGE_2. PGE_2 exerts multiple effects on the immune system such as cytokine release, lymphocyte proliferation and function. PGE_2 has been shown to inhibit differentiation of lymphokine activated killer cells (LAK), suppression of natural killer cell (NK) activity (2-4), and downregulation of a humoral response (5). Furthermore, PGE_2 has been shown to inhibit T cell proliferation in lymphocyte cultures through the downregulation of MHC class II expression on antigen presenting cells (6, 7) and through suppression of cytokine production (8, 9). PGE_2 is produced by cyclooxygenase (COX) mediated oxidation of arachidonic acid. COX, also known as Prostaglandin H synthase, exists in two isoforms, referred to as COX-1 and COX-2. Both isoform are about 60 % homologous within species and all amino acids responsible for the catalysis of PGE_2 are conserved in COX-1 and COX-2. However, it has been suggested that COX-1 and COX-2 have different biological functions: COX-1 which is constitutively expressed in most mammalian tissues possesses house-keeping functions, like cytoprotection of the gastric mucosa (10), renal blood flow and regulation of platelet aggregation (11). COX-1 was found to be overexpressed in 30 of 44 breast tumor tissues compared to normal breast epithelium (12). In contrast, COX-2 is found in normal tissue only after induction with pro-inflammatory or mitogenic stimuli (13, 14). Enhanced expression of COX-2 has been reported in a few metastatic breast cancer cell lines (15) resulting in high levels of PGE_2 production by these cells. Elevated PGE_2 levels have also been found in at least some breast cancer cells (16, 17). However, the role of COX expression and PGE_2 production on anti-tumor immune responses remains poorly understood.

In this study we investigated the involvement of breast cancer derived PGE_2 on B7-1 induced T cell responses. We demonstrated that the inhibition of COX activity in B7-1 expressing MDA-MB 231 cells reconstituted T cell proliferation. Furthermore, the overexpression of COX-1 on B7-1 expressing breast epithelial cells and melanoma cells inhibited T cell responses. Our data suggest that inhibition of COX may potentate B7-1 induced immune responses against breast cancer, making a B7-1 immunotherapy a feasible application in breast cancer. Therefore, a combination therapy of B7-1 transfer and COX inhibition should be considered.

Materials and Methods

Cell lines

The murine mammary carcinoma SCK cell line was provided by Dr. G. Rhee (University of Maryland, MD) and cultured in RPMI medium (Gibco, Grand Island, NY) containing 10% FBS, 1% penicillin/streptomycin (Gibco, Grand Island, NY). MCF-7 cells, MDA-MB 231 cells and the human normal breast epithelial cell line HBL-100 were obtained from the American Type Culture Collection (Rockville, MD). MDA-MB 231 cells were cultured in 50% DMEM medium, 50% F-12 medium supplemented with epidermal growth factor (Sigma, 20 ng/ml), cholera toxin (Sigma, 0.1 μg/ml), insulin (2 μg/ml) and hydrocortisone (0.5 μg/ml). MCF-7 cells were cultured in DMEM medium, 10% FBS and insulin (10 μg/ml) and HBL-100 cells grew in McCoy's 5a medium, 10% FBS. The growth medium of the murine K1735 melanoma cell line (Dr. J. P. Allison, University of California Berkley, CA) was DMEM medium containing 10% FBS and 1% penicillin/streptomycin (Gibco, Grand Island, NY). The human metastatic melanoma cell line WM9 (gift from Dr. M. Herlyn, Wistar Institute, Philadelphia, PA) was cultured in MCDB-153 medium (Sigma, St. Louis, MO) containing 20% L-15, 2% FBS (Gibco, Grand Island, NY) and 0.01 mg/ml insulin (Sigma). All tumor cell lines used in this study did not express cell surface B7-1 as determined by flow cytometry. The retrovirus packaging Bosc cell line was provided by Dr. W. Kerr (University of Pennsylvania, PA) and cultured in DMEM medium, 10% FBS, 1% P/S.

Construction of a retroviral vector expressing Cox-1 and stable expression of Cox-1 on tumor cells

The human Cox-1 cDNA was excised from the Bluescript BShCox-1 KS + plasmid (Dr. G. Fitzgerald, University of Pennsylvania) with BamHI and subcloned into BamHI site of the retroviral vector pLXSP to create pLXSPCOX-1. The right orientation was confirmed by restriction digests. Bosc packaging cells were transfected with pLXSPCOX-1 by the calcium phosphate method. The retroviral supernatant was harvested 48 hours post transfection, filtered through 0.45 μM filter, supplemented with polybrene (8 μg/ml) and was added to subconfluent SCK cells, SCKmB7 cells, K1735 cells and K1735mB7 cells. After 5 hours fresh medium was added and cells were put under selection (puromycin 4 μg/ml) the next day. Every three days fresh selection medium was added and single colonies of puromycin-resistant cells (SCKCOX-1

7

cells, SCKmB7mCOX-1 cells, K1735mCOX-1 and K1735mB7mCOX-1 cells) were expanded. The cells that produced the highest levels of PGE_2 were used for tumorigenicity studies.

The effect of COX-1 on tumorigenicity

Untransduced K1735 cells, K1735mB7, K1735mCOX-1 and K1735mB7mCOX-1 cells ($1x10^6$ cells/mouse) were injected subcutaneously into the right flank of immunocompetent C3H mice. Each group consisted of 8 mice. The animals were observed for tumor outgrowth every other day and tumor sizes were monitored. As soon as a tumor reached a size of 1cm in largest diameter the mouse was euthanized.

Inhibition of COX activity

Untransduced and transduced MDA-MB 231 cells were cultured for 2 days in 60 mm culture dishes in growth medium containing the Cox-2 specific inhibitor NS-398 (Cayman, Ann Arbor, MI) or indomethacin (Sigma). NS-398 and indomethacin were dissolved in ethanol and diluted in RPMI, 10% FBS to a final concentration of 100 μM or 0.5 mg/ml, respectively.

PGE_2 competitive enzyme-immunoassay (PGE_2-EIA)

The concentration of PGE_2 in the CM from MDA-MB 231 cells or from the co-culture of T cells and tumor cells was detected by a competitive enzyme linked immunoassay (EIA) kit (Cayman, Ann Arbor, MI) following the manufacturer's protocol.

Adenoviral transduction

Tumor cells and human breast epithelial HBL-100 cells ($3x10^6$ cells) were transduced in vitro with an adenovirus expressing B7-1 (Ad.hB7-1), an adenovirus expressing the reporter gene β-galactosidase (Ad.lacZ), an adenovirus expressing COX-1 (AdCOX-1, Dr. G. Fitzgerald, University of Pennsylvania) or an adenovirus without transgene (AdBgl2) in growth medium containing 2% FBS. The next day, fresh growth medium was added and transgene expression was assessed by flow cytometry three days after transduction. Generally, a multiplicity of infection (MOI) of 100 plaque forming units (pfu) per cell was used unless indicated otherwise.

Flow cytometry

The expression of B7-1 before and after adenoviral transduction was detected using the BB-1/B7-1 mAb (Becton-Dickinson, Sunnyvale, CA) followed by a fluorescein isothiocyanate (FITC) labeled goat anti-mouse secondary antibody (Pharmingen, San Diego, CA). Ad.lacZ transduced tumor cells were incubated with fluorescein di-β-D-galactopyranoside (FDG, Molecular Probes, Eugene, OR) for one minute at 37°C followed by incubation on ice for 30 minutes. Cellular fluorescence (10,000 live cells/condition) were analyzed using a FACScan flow cytometer (Becton Dickinson).

Isolation of human peripheral blood mononuclear (MN) cells and purified T cells

Peripheral blood was collected in heparin tubes (Becton Dickinson, Franklin Lakes, NJ), diluted with an equal volume of PBS and underlaid with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). After centrifugation at 3,000 rpm for 20 minutes at room temperature, the MN cell layer at the interface was removed, washed twice, and cell number was adjusted in RPMI medium, 10% heat inactivated FBS. Human T cells were isolated from the peripheral lymphocyte fraction by negative selection. Lymphocytes were incubated with an antibody mixture consisting of mouse mAbs to human CD14 to eliminate macrophages, CD16 to eliminate NK cells, CD19 to eliminate B cells and to MHC class II to eliminate MHC class II expressing cells (gift from Dr. L. A. Turka, University of Pennsylvania, PA) for one hour on ice. After washing, cells were resuspended in RPMI medium containing 2% FBS and incubated with BioMag® goat anti-mouse IgG magnetic beads (Perseptive Diagnostics, Cambridge, MA). After magnetic separation, the supernatant containing the T cell fraction was washed twice and counted. The functional purity of the T cell fraction was verified by their failure to proliferate in response to phytohemagglutinin (PHA) alone.

[^{3}H]-thymidine incorporation assay

WM9 and MCF-7 cells (1×10^6) were treated with 100 µg mitomycin C (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 45 minutes at 37°C in serum-free RPMI medium. Cells were washed twice in RPMI medium and resuspended in RPMI medium containing 10% heat-inactivated FBS. Tumor cells (2.5×10^4 cells/well) and T cells or lymphocytes (1×10^5 cells/well) were co-cultured in 96-well round bottomed plates in RPMI medium containing 10% FBS. PHA

(Boehringer Mannheim Biochemicals) was used at a concentration of 5 µg/ml. Phorbol myristate acetate (PMA, Calbiochem, La Jolla, CA) and ionomycin (Sigma) were added at a final concentration of 10 ng/ml and 360 ng/ml, respectively. After five days, cells were pulsed with one µCi [^{3}H]-thymidine (Dupont NEN, Boston, MA) and harvested 18 hours later. Thymidine incorporation was measured in cpm in a liquid scintillation counter (Wallac, Gaithersbury, MD). Each experiment consisting of quadruplicate samples was performed at least twice.

Results

Tumorigenicity of tumor cells expressing murine B7-1 and murine COX-1.

The expression of B7-1 on tumor cells has been shown to induce T cell mediated immune responses in vivo resulting in tumor rejection (1). We sought to investigate whether the expression of COX-1 on tumor cells can alter a B7-1 mediated immune response in vivo. We transduced SCK and B7-1 expressing SCK cells (SCKmB7-1) with a retrovirus expressing murine COX-1. Since mammary carcinoma cells are less immunogenic and therefore not susceptible to B7-1 immunotherapy we also created COX-1 stable transfectants of the murine K1735 cells and K1735mB7-1 cells (K1735COX-1 and K1735B7-1COX-1, respectively). The COX-1 transfected cells were clonally expanded and the PGE_2 secretion into the supernatant was confirmed by PGE_2-EIA. None of the SCK clones cultured in selection medium for several weeks produced PGE_2 levels at significant amounts. Most of the K1735mCOX-1 and K1735mB7-1mCOX-1 clones released PGE_2 into the supernatant and two clones producing the highest amount of PGE_2 were used for animal studies. The K1735mCOX-1 clone produced 1208 pg/ml PGE_2 and the K1725mB7-1mCOX-1 clone produced 917 pg/ml PGE_2. When C3H mice were injected subcutaneously with untransduced K1735 cells, K1735mB7-1 or B7-1/COX-1 expressing K1735 cells, tumors appeared in some animals on day 8 and all animals developed tumors by day 13 (Fig. 1). The tumors grew progressively and animals had to be euthanized by day 24 due to tumor burden. There was no difference in tumor appearance and tumor growth rate between groups.

Co-culture of murine T cells and K1735 cells retrovirally transduced to express B7-1 and/or COX-1.

Since B7-1 expression on K1735 cells did not protect syngeneic animals from tumor growth, we next tested whether we can detect stimulation of T cells in vitro. Untransduced and B7-1/COX-1 expressing K1735 cells were co-cultured with allogeneic T cells (Fig. 2). In the absence of mitogen K1735mB7-1 cells failed to stimulate T cells to proliferate. Similarly, T cells did not proliferate in the presence of B7-1 and COX-1 expressing K1735 cells or in the presence of untransduced tumor cells. There was no significant difference in thymidine uptake between T cells co-cultured with untransduced or transduced K1735 cells. T cells proliferated when Con A was added, indicating that T cells were alive and responsive.

Indomethacin treatment of breast cancer cells restored lymphocyte proliferation.

We have previously shown that depletion of PGE_2 from MCF-7 CM restored the mitogenic response to mononuclear (MN) cells (last annual report). However, MN cultures are mixed cell cultures with endogenous sources of B7-1 and PGE_2. We investigated whether breast cancer derived PGE_2 contributes to the suppresssion of B7-1 dependent T cell responses irrespective of other cell types in MN cultures. Purified T cell co-culture assays were performed using MDA-MB 231 cells that normally produced high amounts of PGE_2. We sought to test whether the treatment of B7-1 expressing MDA-MB 231 cells with COX inhibitors reconstituted T cell proliferation. Therefore, MDA-MB 231 cells were transduced to express B7-1 and treated with indomethacin or NS398, a COX-2 specific inhibitor. As a control, MDA-MB 231 cells and T cells were co-cultured in ethanol containing medium since ethanol served as a solvent for the COX inhibitors. B7-1 expressing MDA-MB 231 cells produced 312 pg/ml PGE_2 and there were no detectable levels of PGE_2 after the treatment with either COX inhibitor by EIA. Untreated or untransduced MDA-MB 231 cells failed to stimulate allogeneic T cells in the absence of PHA (Fig. 3). However, T cells were stimulated to proliferate when co-cultured with B7-1 expressing, indomethacin treated tumor cells in the presence of PHA. In contrast, there was no thymidine incorporation by T cells co-cultured with ethanol treated or NS398 treated, B7-1 expressing MDA-MB 231 cells or with unmodified MDA-MB 231 cells in PHA containing medium (Fig. 3). These results indicate that the inhibition of COX activity and PGE_2 production by indomethacin restored T cell proliferation although this effect was observed only in the presence of PHA. Thus, breast cancer derived PGE_2 appeared to inhibit B7-1 induced T cell proliferation.

Adenoviral transduction of breast epithelial cells and tumor cells.

Rather than reducing PGE_2 production we attempted to confer COX-1 expression and PGE_2 production in breast epithelial and WM9 cells to further support a role of PGE_2 on B7-1 dependent T cell responses. The epithelial cell line HBL-100 and some tumor cells (SUM 185 cells, WM9 cells) did not secrete PGE_2 at detectable levels by EIA and none of the cell lines expressed B7-1. In order to convert these cells PGE_2 producing and B7-1 positive cells, we transfected the cell lines with AdCOX-1 or AdhB7-1 alone or with the combination of these adenoviruses at different multiplicities of infection (MOIs). A successful transduction with

AdhB7-1 was determined by flow cytometry for B7-1 expression. To assess whether adenovirally transduced COX-1 was active, we tested the ability of transduced breast epithelial cells and transduced melanoma cells to produce PGE_2. Breast epithelial cells produced 15 pg/ml, WM9 cells normally released 29 pg/ml and SUM185 cells did not produce PGE_2 at detectable levels (Table 1). Upon transfection with AdCOX-1 the PGE_2 secretion could be enhanced dose-dependently in HBL-100 and WM9 cells up to 23-fold after transduction. Low level PGE_2 production by SUM185 cells could be induced through the transduction with AdCOX-1 (50 pg/ml). When AdhB7-1 and AdCOX-1 were used in combination to transduce HBL-100 or WM9 cells, a MOI of 50 was sufficient to render all cells B7-1 positive (>84%). To induce high level PGE_2 production by WM9 and HBL-100 cells AdCOX-1 was used at MOI of 1000 and 500, respectively. In summary, a transfer of B7-1 and COX-1 on HBL-100 and WM9 cells was achieved by using a combination of AdhB7-1 and AdCOX-1.

The expression of COX-1 on HBL-100 breast epithelial cells inhibits a mitogenic response of T cells.

Next, we addressed whether adenoviral delivered B7-1 and COX-1 was functional. We postulated that the transfer of B7-1 on HBL-100 cells induced the proliferation of T cells in response to PHA and that the co-expression of COX-1 abrogated this effect. We transduced HBL-100 cells with AdCOX-1 in combination with AdhB7-1. T cells co-cultured with B7-1 expressing HBL-100 cells in the presence of PHA proliferated as indicated by thymidine updtake (Fig. 4). However, when COX-1 was expressed on HBL-100 cells in addition to B7-1 the proliferative response of the T cells was inhibited. This result indicates that a single modification (COX-1 expression) can abrogate a B7-1 dependent mitogenic T cell response.

Tumor cell derived PGE_2 inhibits B7-1 dependent T cell proliferation.

To support the hypothesis that tumor cells block B7-1 dependent T cell mediated immune responses through the production of PGE_2, we generated PGE_2 producing and B7-1 positive melanoma WM9 cells by transduction with AdCOX-1 and AdhB7-1. Proliferation assays using T cells co-cultured with modified WM9 cells were performed and the level of PGE_2 in the five-day co-culture medium was determined by PGE_2-EIA. T cells were stimulated to proliferate by the co-culture with B7-1 expressing WM9 cells in the absence or presence of PHA (Fig. 5).

However, the proliferation of T cells was inhibited when incubated with WM9 cells transduced to express B7-1 and COX-1. There was a four fold increase of PGE_2 in the five days supernatant of T cells co-cultured with B7-1, COX-1 expressing WM9 cells (538 pg/ml) as compared to the supernatant from T cells co-cultured with B7-1 expressing WM9 cells (Table 2). T cells co-cultured with AdB7-1, AdBgl2 infected WM9 cells produced 95 pg/ml PGE_2. The allogeneic T cell response of B7-1 expressing WM9 cells was completely restored by the co-culture of T cells with WM9 cells transduced with a mixture of AdhB7-1 and AdBgl2. These results clearly indicate that the expression of COX-1 and the resulting production of PGE_2 by WM9 cells inhibited B7-1 induced T cell proliferation.

cells	virus	MOI	% pos for B7-1 expression	PGE$_2$ (pg/ml)
WM9	AdhB7-1	0 10 50 100	5% 55% 95% 99%	
WM9	AdCOX-1	0 100 500 1000 5000		29 pg/ml 61 pg/ml 180 pg/ml 462 pg/ml 701 pg/ml
WM9	AdhB7/ AdCOX-1 AdhB7/ AdBgl2	50/ 1000 50/ 1000	93% 90%	361 < detection limit
SUM185	AdCOX-1	0 100 500 1000		9 15 15 50
HBL-100	AdhB7-1 AdCOX-1	10 50 100 50 100 500	85% 93% 95%	 15 86 387
HBL-100	AdhB7/ AdCOX-1	50/ 50 50/ 100 50/ 500	86% 84% 95%	30 140 355

Table 1: B7-1 expression and PGE$_2$ production before and after transduction of human metastatic WM9 cells, human SUM 185 breast cancer cells and human HBL-100 breast epithelial cells with AdhB7 and/or AdCOX-1. B7-1 expression was determined by flow cytometry and PGE$_2$ production was assessed by PGE$_2$-EIA.

cells	T cells	PHA	PGE$_2$ (pg/ml)
WM9	/	/	57
	+	/	29
	+	+	96
WM9AdhB7	/	/	91
	+	/	139
	+	+	165
WM9AdhB7/AdCOX-1	/	/	500
	+	/	538
	+	+	692
WM9AdhB7/AdBgl2	/	/	103
	+	/	95
	+	+	205
MN cells	/	/	63
	/	+	730
T cells	/	/	7
	/	+	9

Table 2: PGE$_2$ levels in the five day supernatants of T cells co-cultured with untransduced and transduced WM9 cells.
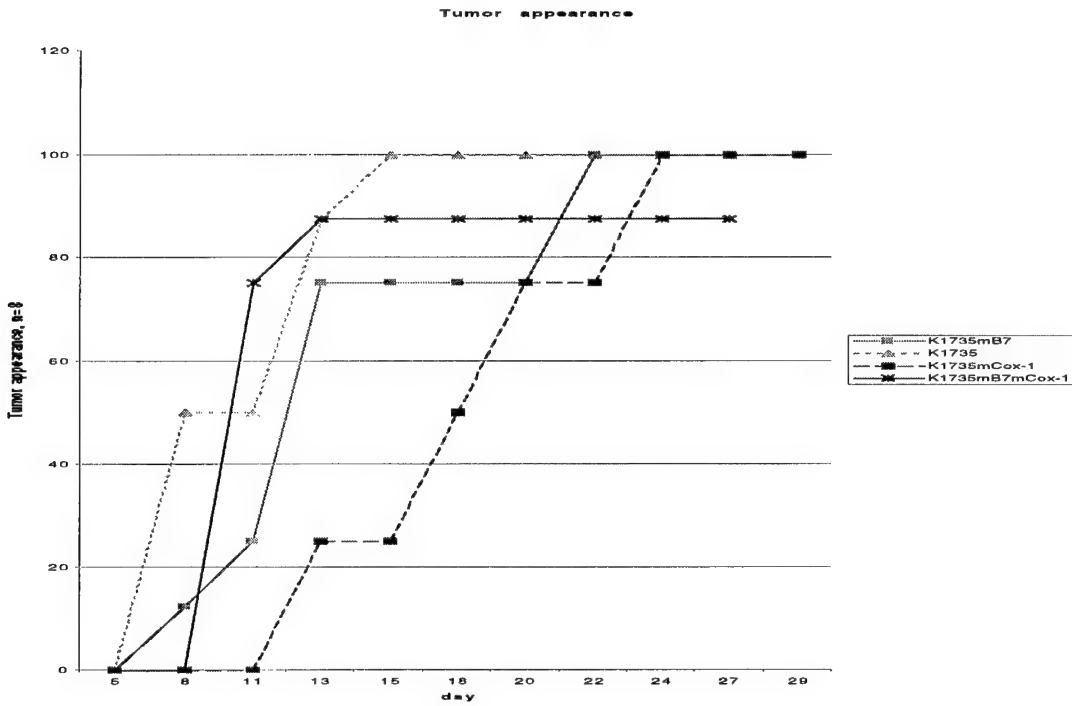
Fig. 1: Tumorigenicity of K1735mB7-1 and K1735mB7-1mCOX-1 cells. Mice were inoculated with live untransduced K1735 cells and retrovirally transduced K1735 cells expressing B7-1 and/or COX-1 in the right flank (8 mice per group). Mice were monitored every other day for tumor appearance. There was no difference in tumor appearance and tumor growth rate between groups.
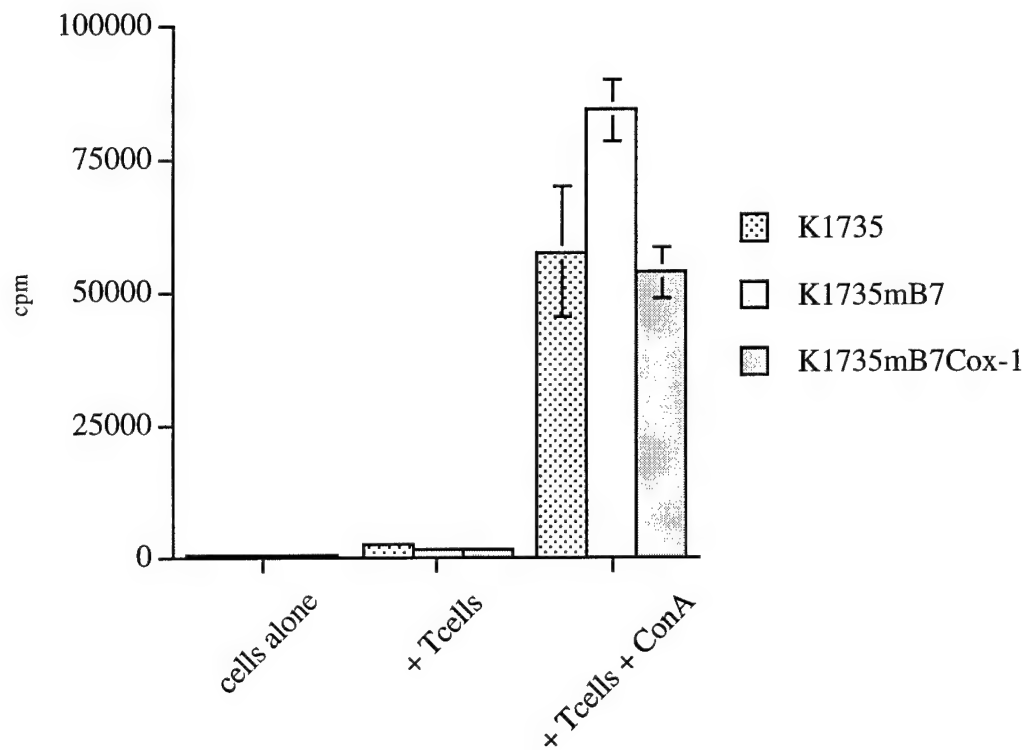
Fig. 2: Co-culture of T cells with K1735 cells expressing B7-1 and/or COX-1. Data were obtained as a mean of quadruplicates and error bars represent the standard deviation. There is no difference in thymidine incorporation by murine T cells co-cultured with K1735mB7-1 cells as compared to untransduced or B7-1 and COX-1 expressing cells.
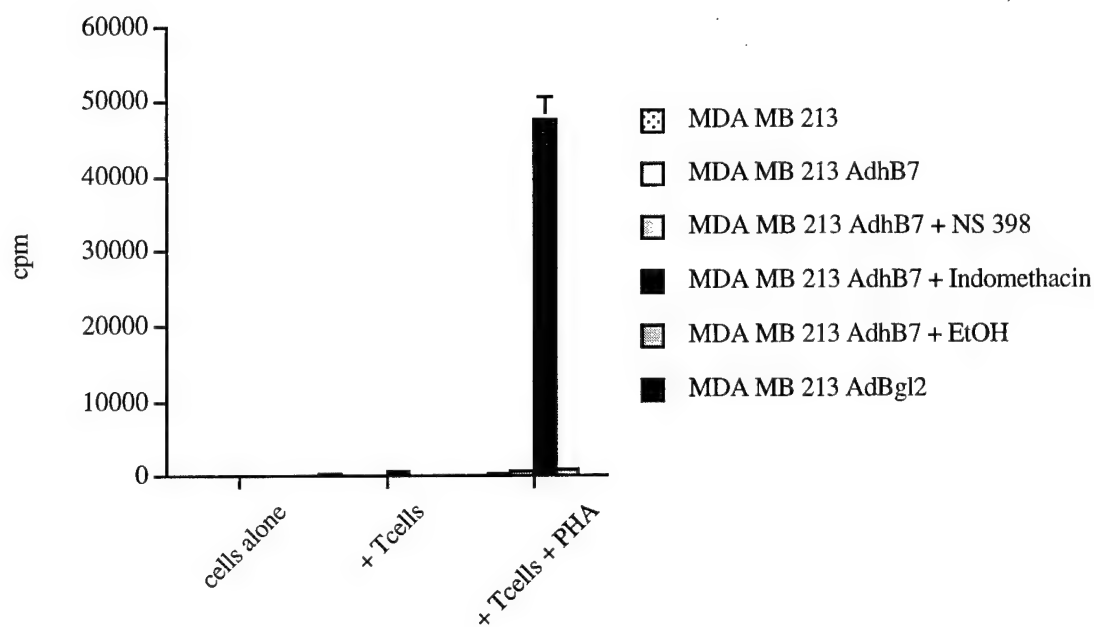
Fig. 3: Indomethacin treatment of B7-1 expressing MDA-MB 231 cells restored the proliferation of T cells. [³H]Thymidine incorporation by T cells co-cultured with B7-1 expressing MDA-MB 231 cells after the treatment with indomethacin, NS 398 or ethanol. Data were obtained as a mean of quadruplicates and error bars represent the standard deviation. In the presence of PHA indomethacin treatment of B7-1 expressing MDA-MB 231 cells abrogated T cell proliferation.
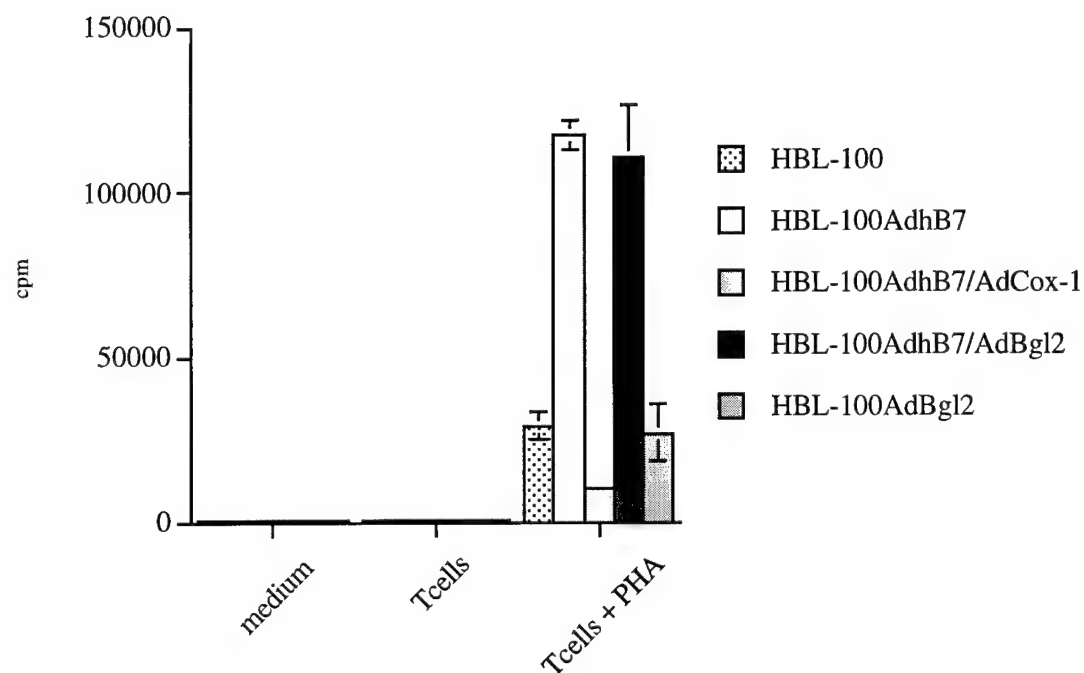
Fig. 4: The expression of COX-1 on HBL-100 cells inhibited the proliferative response of T cells to PHA. [³H]Thymidine incorporation by T cells co-cultured with HBl-100 cells transduced with AdhB7 and/or AdCOX-1. B7-1 exppressing cells and PHA stimulated T cells to proliferate, whereas the co-expression of COX-1 inhibited T cell proliferation in this setting.
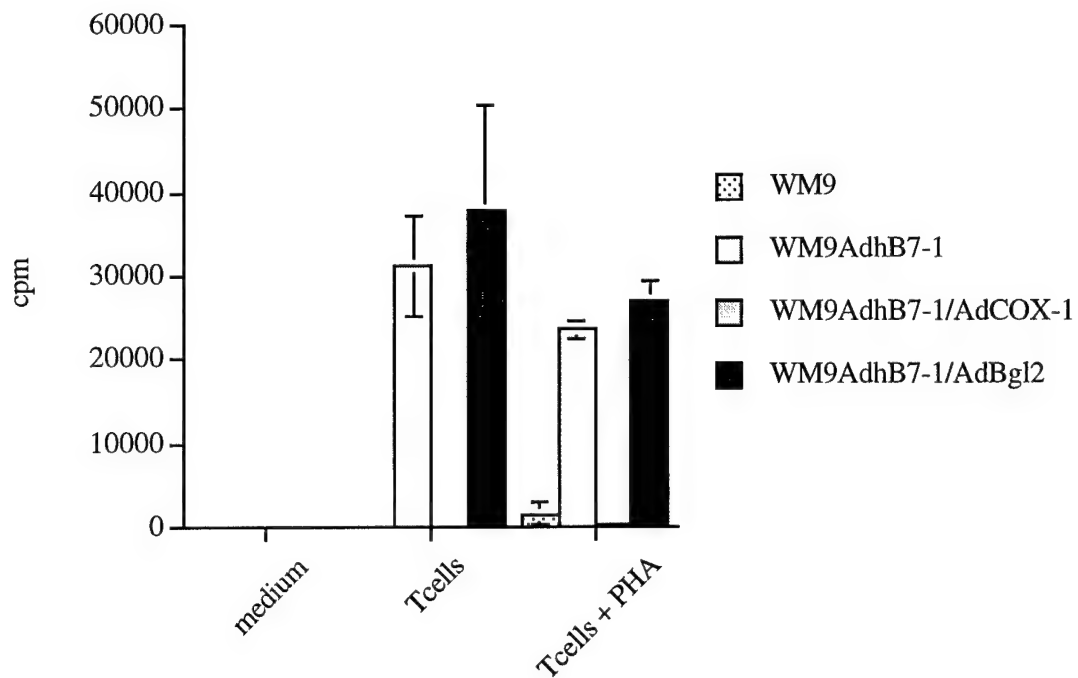
Fig. 5: [^{3}H]Thymidine incorporation by T cells co-cultured with WM9 cells co-expressing COX-1 and B7-1. Data were obtained as a mean of quadruplicates and error bars represent the standard deviation. The proliferation of T cells in response to B7-1 expressing WM9 cells was inhibited by the co-expression of COX-1 on the tumor cells.

Discussion

B7-1 immunotherapy has been reported to be effective in immunogenic tumors (1). Tumor cells are generally termed "immunogenic" when they express antigens that result in the rejection of tumor cells by syngeneic animals previously immunized with the irradiated parental tumor cells (1). Accordingly, non-immunogenic tumors are rejected when similarly tested. However, there is no consensus about the parameters that render tumor cells more or less immunogenic. Breast cancer cells are known as non-immunogenic tumors and thus escape immune surveillance. We investigated whether the production of PGE_2 by mammary carcinoma cells rendered them non-immunogenic. The significance of the inhibitory effects of PGE_2 in vivo remains to be tested. In our hands mice injected with B7-1 and COX-1 expressing K1735 and control mice developped tumors at the same time and growth rate. The lack of immunogenicity of K1735B7-1 cells was most likely due to the loss of B7-1 expression. Although cells were cultured in selection medium only 35% of the cells expressed B7-1 as determined by flow cytometry. Thus, the number of B7-1 expressing K1735 cells was not sufficent to induce an anti-tumor immune response.

Here, we have demonstrated that in contrast to B7-1 transduced human melanoma cells, B7-1 immunotherapy using breast cancer cells failed to induce T cell proliferation in vitro due to the secretion of PGE_2. The significance of tumor-derived PGE_2 to T cell growth inhibition is highlighted by recent reports demonstrating therapeutic efficacy in the prevention and treatment of breast cancer by non-steroidal anti-inflammatory drugs (NSAID) including indomethacin itself (3). Importantly, indomethacin reduced PGE_2 production by inhibiting the enzymatic activity of the COX-1 and COX-2 which were rate-limiting enzymes in the PGE_2 biosynthetic pathway. Indomethacin has also demonstrated efficacy in the treatment of mouse mammary carcinoma cells. Mice injected with mammary adenocarcinoma C3-L5 cells received long-term indomethacin therapy on day 15 followed by two rounds of IL-2 administration for five days. Regression of primary tumors, reduction of lung metastases and prolonged survival were observed in the group receiving the combination therapy as opposed to the group receiving IL-2 treatment alone. Furthermore, the long-term intake of indomethacin in combination with IL-2 was shown to activate tumoricidal lymphocytes in situ (3). We have previously shown (last annual report) that indomethacin treatment of MCF-7 cells reduced PGE_2 production and

partially alleviated inhibition of MN cell proliferation. Indomethacin did not completely remove the inhibitory effect that is consistent with the presence of residual PGE_2 that could be detected by mass spectometry. Here, we demonstrate that indomethacin treatment of B7-1 expressing MDA-MB 231 cells inhibited PGE_2 production and reconstituted their ability to stimulate T cell proliferation. This, accompanied by the PGE_2 depletion data (last annual report), suggests that PGE_2 contributed to tumor derived immunosuppression.

The effect of PGE_2 on mitogenic lymphocyte responses is reported before (5, 18). However, the effect of PGE_2 on B7-1 dependent T cell proliferation, an important event in the induction of an anti-tumor immune response, has not been studied in great detail. We have previously shown that breast cancer-derived PGE_2 suppressed the lymphoproliferative response to mitogens (last annual report). This experimental setting, however, did not take into account that lymphocytes are a mixed cell population consisting of B7-1 expressing cells as well as PGE_2 producing cells. We next sougt to investigate the role of PGE_2 on immune responses in a more defined system with no endogenous source of B7-1 or PGE_2. Here, we were able to show that the production of PGE_2 specifically inhibited B7-1 induced T cell proliferation. First, B7-1 expressing MDA-MB 231 cells did not secrete PGE_2 at detectable levels after indomethacin treatment and stimulated T cells to proliferate when PHA was present. Second, the co-expression of B7-1 and COX-1 on breast epithelial cells inhibited the response of T cells to PHA. Finally, in the melanoma system the sole modification of co-expression of COX-1 on B7-1 expressing WM9 cells was sufficient to inhibit an allogeneic T cell response. Thus, the expression of COX-1 is necessary and sufficient to inhibit B7-1 dependent T cell responses in vitro. Therefore, a tumor vaccine based on B7-1 expressing breast cancer cells may benefit from the inhibition of COX activity.

Conclusion

In summary, the use of a B7-1 expressing breast cancer based vaccine is not efficient at inducing T cell proliferation through the prodution of PGE_2 by the tumor cells. Most breast cancer cells secrete PGE_2 and their CM inhibits mitogenic and B7-1 induced T cell proliferation. The depletion of PGE_2 from MCF-7 CM restored the mitogenic response of MN and indomethacin treatment of B7-1 expressing MDA-MB 231 cells restored the proliferative response of T lymphocytes to PHA. MCF-7 CM blocked the proliferation of T cell stimulated with B7-1 expressing melanoma cells and removal of PGE_2 from CM reversed this effect. Furthermore, the transduction of B7-1 expressing melanoma cells with AdCOX-1 resulted in PGE_2 secretion and marked inhibition of T cell proliferation. Together, these results indicate that the efficacy of B7-1 based vaccines in breast cancer may benefit from antagonizing the effects of tumor-derived PGE_2. The combination of inhibiting PGE_2 production and transfer of B7-1 expression on tumor cells abrogated T cell proliferation and may form a basis for an immunotherapy against breast cancer.

References

1. Chen, L., McGowan, P., Ashe, S., Johnston, J., Li, Y., Hellstrom, I., and Hellstrom, K. E. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity, Journal of Experimental Medicine. *179:* 523-32, 1994.

2. Brunda, M. J., Herberman, R. B., and Holden, H. T. Inhibition of murine natural killer cell activity by prostaglandins, Journal of Immunology. *124:* 2682-7, 1980.

3. Lala, P. K., Parhar, R. S., and Singh, P. Indomethacin therapy abrogates the prostaglandin-mediated suppression of natural killer activity in tumor-bearing mice and prevents tumor metastasis, Cellular Immunology. *99:* 108-18, 1986.

4. Skibinski, G., Kelly, R., Harrison, C., McMillan, L., and James, K. Relative immunosuppressive activity of human seminal prostaglandins., Journal of Reproductive Immunology. *22:* 185-95, 1992.

5. Miao, D., Skibinski, G., and James, K. The effects of human seminal plasma and PGE2 on mitogen induced proliferation and cytokine production of human splenic lymphocytes and peripheral blood mononuclear cells, Journal of Reproductive Immunology. *30:* 97-114, 1996.

6. Valitutti, S., Castellino, F., Aiello, F. B., Ricci, R., Patrignani, P., and Musiani, P. The role of arachidonic acid metabolite PGE2 on T cell proliferative response, Journal of Clinical & Laboratory Immunology. *29:* 167-73, 1989.

7. Laning, J. C., Isaacs, C. M., and Hardin-Young, J. Normal human keratinocytes inhibit the proliferation of unprimed T cells by TGFbeta and PGE2, but not IL-10, Cellular Immunology. *175:* 16-24, 1997.

8. Murray, J. L., Dowd, J., and Hersh, E. M. In vitro inhibition of interleukin-2 production by peripheral blood lymphocytes from stage III melanoma patients by prostaglandin E2: enhancement of lymphocyte proliferation by exogenous interleukin-2 plus indomethacin, Journal of Biological Response Modifiers. *5:* 12-9, 1986.

9. Vercammen, C. and Ceuppens, J. L. Prostaglandin E2 inhibits human T-cell proliferation after crosslinking of the CD3-Ti complex by directly affecting T cells at an early step of the activation process, Cellular Immunology. *104:* 24-36, 1987.

10. Kargman, S., Charleson, S., Cartwright, M., Frank, J., Riendeau, D., Mancini, J., Evans, J., and O'Neill, G. Characterization of Prostaglandin G/H Synthase 1 and 2 in rat, dog, monkey, and human gastrointestinal tracts, Gastroenterology. *111:* 445-54, 1996.

11. Vane, J. R. Purity and stability of synthetic peptides such as angiotensins and kinins, Nature. *230:* 382, 1971.

12. Hwang, D., Scollard, D., Byrne, J., and Levine, E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer, Journal of the National Cancer Institute. *90:* 455-460, 1998.

13. DuBois, R. N., Tsujii, M., Bishop, P., Awad, J. A., Makita, K., and Lanahan, A. Cloning and characterization of a growth factor-inducible cyclooxygenase gene from rat intestinal epithelial cells, American Journal of Physiology. *266:* G822-7, 1994.

14. Hempel, S. L., Monick, M. M., and Hunninghake, G. W. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes, Journal of Clinical Investigation. *93:* 391-6, 1994.

15. Liu, X. H. and Rose, D. P. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines, Cancer Research. *56:* 5125-7, 1996.

16. Watson, J. and Chuah, S. Y. Technique for the primary culture of human breast cancer cells and measurement of their prostaglandin secretion, Clinical Science. *83:* 347-52, 1992.

17. Yang, C. Y. and Meng, C. L. Regulation of PG synthase by EGF and PDGF in human oral, breast, stomach, and fibrosarcoma cancer cell lines, Journal of Dental Research. *73:* 1407-15, 1994.

18. Baskar, S., Ostrand-Rosenberg, S., Nabavi, N., Nadler, L. M., Freeman, G. J., and Glimcher, L. H. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules, Proceedings of the National Academy of Sciences of the United States of America. *90:* 5687-90, 1993.

Recommendations in relation to the Statement of Work outlined in the proposal

As previously shown murine mammary carcinoma cells and human breast cancer cells used in this study do normally not express B7-1 but can be adenovirally be transduced to express B7-1 (task 1 and 2 of the Statement of Work). There was no growth difference between untransduced and AdmB7-1 transduced mammary carcinoma cells in immunocompetent mice (task 5). Thus, the comparison of the in vivo growth behaviour in immunocompetent mice (task 6) was omitted. Preliminary lymphocyte proliferation data suggested that PGE_2 may suppress T cell responses in vitro. To determine the role of PGE_2 in vivo we carried out immunogenicity studies (proposed in task 8) including a group of mice receiving B7-1 and COX-1 expressing tumor cells. All mice needed to be euthanized due to tumor burden. The induction of T cell mediated immune responses by B7-1 expressing breast cancer cells was investigated in great detail (task 9). B7-1 expressing breast cancer cells failed to stimulate T cells to proliferate (task 9). Therefore, we hypothesized that human breast cancer cells produce immune inhibitory factors. Thymidine incorporation assays revealed that the CM from most breast cancer cell lines inhibited T cell proliferation and PGE_2 could be identified as a major mediator for the inhibition of B7-1 induced T cell proliferation (task 9).

Appendix

Bulleted list of key research accomplishments

- Mammary carcinoma cells and breast cancer cells can be transduced with adenovirus expressing B7-1 at high efficiency.
- B7-1 expressing breast cancer cells failed to induce T cell proliferation in vivo and in vitro.
- CM from breast cancer cells inhibited a mitogenic response of lymphocytes.
- Soluble TGFβ did not account fro the inhibitory effect of MCF-7 CM on lymphocyte proliferation.
- CM from breast cancer cells contained PGE_2 at various amounts.
- Indomethacin treatment of breast cancer cells restored lymphocyte proliferation.
- Removal of PGE_2 from breast cancer CM elicited B7-1-depedent stimulation of MN cells and purified T lymphocytes.
- Breast cancer derived PGE_2 inhibited B7-1-dependent T cell proliferation induced by melanoma cells.
- Melanoma cells transduced to produce PGE_2 blocked B7-1 dependent T cell proliferation.

List of reportable outcomes

1. The work of this study is presented in a manuscript that will be submitted for publication in Cancer Research.

2. Parts of the work funded by the U.S. Army Breast Cancer Research Program grant DAMD17-96-1-6287 was presented as a minisymposium at the American Association for Cancer Research meeting in Philadelphia on April 10-14, 1999.

3. The work presented in this study formed the basis for my Ph.D. thesis and I graduated on November 20, 1998 with honory degree (Magna cum lauda) from the "Justus-Liebig University" in Giessen, Germany. The Ph.D. program at the "Justus-Liebig University" in Giessen, Germany was conducted in affiliation with the University of Pennsylvania.

4.   Based on the skills and training supported by the DAMD17-96-1-6287 award I received and accepted a job offer as an "Associate Scientist" at Cell Genesys Inc., 342 Lakeside Drive, Foster City, CA 94404 starting in October 1999. My work at Cell Genesys will involve the characterization of humoral and cellular immune responses incancer patients receiving treatment with a GM-CSF tumor vaccine.

Please find enclosed a copy of the manuscript submitted to Cancer Research and the abstract submitted to the American Association for Cancer Research summarizing my work supported by the U.S. Army Breast Cancer Research Program grant DAMD17-96-1-6287.

Human breast cancer derived PGE$_2$ inhibits B7-1 induced T cell proliferation
Heike K. E. Boxhorn, Ulrich Rodeck, Ralf Gutzmer, Monika Jost, Stephen L. Eck; Hematology/Oncology Division, Department of Medicine, University of Pennsylvania Medical Center, Philadelphia, PA 19104 (H.K.E.B.,R.G., S.L.E.); Department of Dermatology, Thomas Jefferson University, Philadelphia, PA 19107 (U.R., M.J.)

Expression of the T cell co-stimulatory molecule B7-1 has been shown to induce anti-tumor immunity in several murine tumor models. B7-1 expressing tumor cells have been postulated to directly activate T cells without the need for antigen processing by antigen presenting cells. To evaluate the potential of B7-1 breast cancer immunotherapy we transduced human MCF-7 breast cancer cells to express high levels of B7-1 using an adenoviral expression vector (Ad.hB7-1). The co-culture of B7-1 expressing MCF-7 cells with purified human T cells failed to augment T cell proliferation. Under the same conditions human melanoma cells expressing B7-1 were able to induce T cell proliferation. The lack of T cell stimulation by MCF-7 cells was due to secretion of a soluble factor by MCF-7 cells that strongly inhibited T cell proliferation. Furthermore, we found that seven of eight human breast cancer lines similarly inhibited lymphocyte proliferation. The principal component of the inhibitory effect of MCF-7 cells was identified as prostaglandin E$_2$ (PGE$_2$) using PGE$_2$ depletion experiments. TGFβ is also produced by MCF-7 cells, but had no demonstrable effect on B7-1-dependent T cell proliferation. These results suggest that PGE$_2$ secreted by human breast cancer cells may limit the use of tumor vaccines based on B7-1 expressing breast cancer cells.

Human breast cancer derived PGE$_2$ inhibits B7-1 induced T cell proliferation

Heike K. E. Nesbit[1], Ulrich Rodeck[2], Ralf Gutzmer[1], Monika Jost[2], Stephen P. Ethier[3], Peter McNamara[4], Stephen L. Eck[1]*

[1]Hematology/Oncology Division, Department of Medicine, University of Pennsylvania Medical Center, Philadelphia, PA 19104

[2]Department of Dermatology, Thomas Jefferson University, Philadelphia, PA 19107

[3]Radiation and Cancer Biology Division, Department of Radiation Oncology, University of Michigan, Ann Arbor, MI 48109

[4]Center of Experimental Therapeutics, University of Pennsylvania Medical Center, Philadelphia, PA 19104

*To whom inquiries should be addressed.

Correspondent Footnote:
512 BRB II/III, 421 Curie Blvd.
Philadelphia, PA 19104-6160
Phone: (215) 898-4178
Fax: (215) 573-8606
email: ecks@mail.med.upenn.edu

Abstract

Expression of the T cell co-stimulatory molecule B7-1 has been shown to induce anti-tumor immunity in several murine tumor models. B7-1 expressing tumor cells have been postulated to directly activate T cells without the need for antigen processing by antigen presenting cells. To evaluate the potential of B7-1 breast cancer immunotherapy we transduced human MCF-7 breast cancer cells to express high levels of B7-1 using an adenoviral expression vector (AdB7-1). The co-culture of B7-1 expressing MCF-7 cells with purified human T cells failed to augment T cell proliferation. Under the same conditions human melanoma WM9 cells expressing B7-1 were able to induce T cell proliferation. We hypothesized that the lack of T cell stimulation by B7-1 expressing MCF-7 cells was due to secretion of a soluble factor and sought to identify which factor was responsible for the inhibition of B7-1 induced T cell proliferation. We found that conditioned medium (CM) from eight of nine human breast cancer lines including MCF-7 cells inhibited lymphocyte proliferation. $TGF\beta$ was produced by MCF-7 cells but had no demonstrable effect on the mitogenic response of lymphocytes. However, several lines of evidence suggest that the principal component of the inhibitory effect of MCF-7 and MDA MB 231 cells was prostaglandin E_2 (PGE_2). Depletion of PGE_2 from the supernatant of MCF-7 cells restored not only the mitogenic response of lymphocytes to phytohemagglutinin (PHA) in the presence of MCF-7 CM, whereas MCF-7 CM was able to block the proliferation of T cell stimulated with B7-1 expressing melanoma cells. PGE-depleted MCF-7 CM was no longer able to inhibit T cells in this assay. Furthermore, the transduction of B7-1 expressing melanoma cells with an adenovirus expressing cyclooxygenase-1 (AdCOX-1) resulted in the inhibition of T cell proliferation. Thus, this study provides direct evidence that breast cancer derived PGE_2 blocks B7-1 dependent proliferation of T cells. Therefore, PGE_2 secreted by human breast cancer cells may limit the use of tumor vaccines based on B7-1 expressing breast cancer cells.

2

Introduction

Cancer immunotherapy seeks to elicit and/or restore clinically effective immune responses to tumor antigens. In recent years T cell-mediated immune responses to tumor cells have attracted special attention because they constitute an effective mechanism for destruction of antigen-bearing target cells. In addition, T lymphocytes can differentiate into long-lasting memory cells thus potentially providing lasting protection against tumor recurrence. Importantly, T cells reactive against tumor antigens have been identified in tumor patients (1), however, in the presence of growing tumors, these T lymphocytes do not efficiently destroy tumor cells.

Two signals are required for effective T cell activation: an antigen-specific signal and an antigen-independent, co-stimulatory signal. The antigen-specific signal is provided by the interaction of the T cell receptor (TCR) with peptides presented by major histocompatibility (MHC) proteins. The non antigen-specific signal is generated by co-stimulatory molecules, which include members of the B7 family of proteins, that interact with cognate ligands on T cells (2). The interaction between B7-1 on antigen presenting cells and its counter-receptor CD28 on T lymphocytes plays a key role in the induction of cell-mediated immune responses in part by preventing T cell apoptosis during target cell lysis (3). Importantly, tumor cells typically lack the expression of co-stimulatory molecules. The delivery of antigen-specific signals by tumor cells in the absence of co-stimulatory signals is believed to lead to T cell anergy or apoptosis (2-4). Several groups have shown that expression of B7-1 on murine melanoma cells (5-9) and other tumor cells results in tumor rejection and the induction of protective immunity (5, 6, 8-12). However, B7-1 expression is not as effective in providing protection against poorly immunogenic tumors (13), including murine mammary carcinomas (8). At present, it is not known why B7-1 expression is less effective in certain tumor models.

4

Breast carcinoma cells produce high levels of agents known to alter T cell responses including transforming growth factor β (TGFβ, 14), interleukin 10 (15), and PGE_2 (16). PGE_2 increases the expression of receptors on lymphocytes, promotes immunoglobulin synthesis, inhibits interleukin production, cytotoxicity and lymphocyte proliferation (Phipps RP1999 Immunology today, Roper RL 1994 Adv Prrost Thromb Leuk Res; Coleman RA 1994 Pharmac Rev). Moreover, PGE_2 has been shown to inhibit differentiation of lymphokine activated killer cells (LAK), suppression of natural killer cell (NK) activity (26-28), and downregulation of a humoral response (29). The inhibition of T cell proliferation in lymphocyte cultures by PGE_2 is mediated by the downregulation of MHC class II expression on antigen presenting cells (30-32) and through suppression of cytokine production (33, 34). PGE_2 is produced by COX mediated oxidation of arachidonic acid and has been found in some human breast cancer cell lines. There are two isoforms of COX, designated COX-1 and COX-2. In mouse thymus PGE_2 production plays a critical role in T cell maturation (Rocca B, FitzGerald GA JCI 1999 103, 10). COX-1 is constitutively expressed in most tissues and mediates the synthesis of prostaglandins required for constituative physiological functions, such as maintaining gastrointestinal, kidney and reproductive functions (Subbaramaiah k, Dannenberg Aj, Proc Sci for exp Bio&Med). COX-1 was found to be overexpressed in 30 of 44 breast tumor tissues compared to normal breast epithelium (36). COX-2 expression can be induced by cytokines, growth factors, oncogenes and tumor promoters (Kujubu da J Biol Chem 1991), is reportedly upregulated in a few metastatic breast cancer cell lines (35) and leads to high levels of PGE_2 production by these cells. Given the development of idiopathic chronic suppurative peritonitis and bowel inflammation in COX-2 knockout mice (Morham SF 1995 Cell 83), the involvement of COX-2 in immune cell development is likely and requires detailed investigation. However, the role of COX expression and PGE_2

production on anti-tumor immune responses remains poorly understood with respect to its potentially deleterious effects on anti-tumor immunity.

We have previously reported that adenoviral transfer of B7-1 to human melanoma cells elicits allogeneic T cell responses despite the production of immunosuppressive factors by the tumor cells (37). In the present study we show that B7-1 expression on human breast cancer MCF-7 cells failed to stimulate effective T cell responses in vitro under comparable experimental conditions. The failure of B7-1 expressing breast cancer cells to induce T cell proliferation was due in part to soluble immunosuppressive agents produced by the tumor cells. In addition, we provide evidence that tumor-derived PGE_2, but not TGFβ, was responsible for curtailing T cell proliferation in this experimental setting.

Materials and Methods

Cell lines and culture methods

The human metastatic melanoma cell line WM9 (gift from Dr. M. Herlyn, Wistar Institute, Philadelphia, PA) was cultured in MCDB-153 medium (Sigma, St. Louis, MO) containing 20% L-15, 2% FBS (Gibco, Grand Island, NY) and 0.01 mg/ml insulin (Sigma). The human breast cancer cell lines SUM52PE, SUM149PT, SUM185PE and SUM190PT were produced as previously described (38) and were cultured in Ham's F-12 medium (Gibco) containing 5% FBS, insulin (5 μg/ml) and hydrocortisone (Sigma, 1 μg/ml). All other human breast cancer cell lines and the human normal breast epithelial cell line HBL-100 were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells were cultured in DMEM medium (Gibco) containing 10% FBS, 1% penicillin/streptomycin and insulin (10 μg/ml). MCF-10 and MDA-MB 231 cells were cultured in 50% DMEM medium, 50% F-12 medium supplemented with epidermal growth factor (Sigma, 20 ng/ml), cholera toxin (Sigma, 0.1 μg/ml), insulin (2 μg/ml) and hydrocortisone (0.5

6

μg/ml). The growth medium of BT-20 cells was DMEM medium, 10% FBS, 1% penicillin/streptomycin and insulin (2 μg/ml). BT-474 cells were cultured in L-15 medium (Sigma), 10% FBS, 1% penicillin/streptomycin. None of the tumor cell lines used in this study did express cell surface B7-1 as determined by flow cytometry. WM9 cells were positive for MHC I and II molecules, whereas MCF-7 and MDA-MB 231 cells expressed only MHC I as detected by flow cytometry.

Adenoviral transduction

AdhB7-1, AdhCOX-1 and recombinant adenoviruses containing either the β-galactosidase reporter gene (AdlacZ) or no transgene (AdBgl2) were constructed as previously described (37, 39). WM9 and MCF-7 cells (3×10^6 cells) were transduced in vitro with adenovirus at a multiplicity of infection (MOI) of 100 plaque forming units (pfu) per cell in growth medium containing 2% FBS. To create B7-1 and COX-1 positive WM9 cells, cells were transduced with a mixture of AdB7-1 (MOI of 50) and AdCOX-1 (MOI of 1000). The next day, fresh growth medium was added. Transgene expression was assessed by flow cytometry three days after transduction.

Flow cytometry

The expression of B7-1 before and after adenoviral transduction was detected using the BB-1/B7-1 mAb (Becton-Dickinson, Sunnyvale, CA) followed by a fluorescein isothiocyanate (FITC) labeled goat anti-mouse secondary antibody (Pharmingen, San Diego, CA). AdlacZ transduced tumor cells were incubated with fluorescein di-β-D-galactopyranoside (FDG, Molecular Probes, Eugene, OR) for one minute at 37°C followed by incubation on ice for 30 minutes. Cellular fluorescence (10,000 live cells/condition) was analyzed using a FACScan flow cytometer (Becton-Dickinson). The purity of T cells was confirmed by incubation with an anti-CD3 antibody (Pharmingen) followed by a FITC-anti-mouse antibody (Pharmingen).

Production of conditioned media (CM) by tumor cells

For lymphocyte proliferation assays, supernatants of tumor cells (3×10^6 cells) cultured for 24 hours in 5 ml RPMI medium containing 10% heat inactivated FBS were collected. For the detection of TGFβ or PGE$_2$ in MCF-7 CM by bioassay, MCF-7 cells were cultured under serum-free conditions for 24 hours (40). CM was stored in aliquots at -20°C (or at -80°C when used in bioassays for the detection of PGE$_2$). For the detection of PGE$_2$ in the supernatants of T cells co-cultured with modified tumor cells, supernatants from four wells were combined directly before harvesting the 96-well-plates and assayed by bioassay.

Isolation of human peripheral blood mononuclear (MN) cells and purified T cells

Peripheral blood was collected in heparin tubes (Becton-Dickinson, Franklin Lakes, NJ), diluted with an equal volume of PBS and underlaid with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). After centrifugation at 3,000 rpm for 20 minutes at room temperature, the MN cell layer at the interface was removed, washed twice, and cell number was adjusted in RPMI medium containing 10% heat inactivated FBS. Human T cells were isolated from the peripheral lymphocyte fraction by negative selection. Lymphocytes were incubated with an antibody mixture consisting of mouse mAbs to human CD14 to eliminate macrophages, CD16 to eliminate NK cells, CD19 to eliminate B cells and MHC II expressing cells (gift from Dr. L. A. Turka, University of Pennsylvania, PA) for one hour on ice. After washing, cells were resuspended in RPMI medium containing 2% FBS and incubated with BioMag® goat anti-mouse IgG magnetic beads (Perseptive Diagnostics, Cambridge, MA). After magnetic separation, the supernatant containing the T cell fraction was washed twice and counted. The functional purity of the T cell fraction was verified by their failure to proliferate in response to PHA alone and by anti-CD3 staining using flow cytometry analysis (>97% CD3 positive) prior to [^{3}H]-thymidine incorporation assay.

8

[^{3}H]-thymidine incorporation assay

MDA-MB 231 cells were transduced with AdB7-1 and treated with indomethacin (Sigma) at a concentration of 100 μg/ml or with NS398 (Cayman, Ann Arbor, MI) at a concentration of 10^{-4} M for 24 hours. Indomethacin and NS398 were dissolved in ethanol. Untransduced and transduced tumor cells (1×10^6) were then treated with 100 μg mitomycin C (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 45 minutes at 37°C in serum-free RPMI medium. Cells were washed twice in RPMI medium and resuspended in RPMI medium containing 10% heat-inactivated FBS. Tumor cells (2.5×10^4 cells/well) and T cells (1×10^5 cells/well) were co-cultured in 96-well round bottomed plates in RPMI medium containing 10% FBS. For MN proliferation assays, MN cells (1×10^5 cells/well) were cultured in 96-well round bottomed plates in RPMI medium containing 10% FBS. PHA (Boehringer Mannheim Biochemicals) was used at a concentration of 5 μg/ml. Phorbol myristate acetate (PMA, Calbiochem, La Jolla, CA) and ionomycin (Sigma) were added to a final concentration of 10 ng/ml and 360 ng/ml, respectively. After five days, cells were pulsed with one μCi [^{3}H]-thymidine (Dupont NEN, Boston, MA) and harvested 18 hours later. Thymidine incorporation was measured in counts per minute (cpm) in a liquid scintillation counter (Wallac, Gaithersbury, MD). The stimulation index (SI) was calculated as follows: mean cpm of untransduced or transduced tumor cells co-cultured with T cells − mean cpm of untransduced or transduced tumor cells alone / mean cpm of T cells alone. Inhibition of [^{3}H]-thymidine incorporation was calculated as follows: % inhibition = 100% - (100% x cpm of PMA/ionomycin stimulated MN cells incubated with sample CM at a dilution of 1:4 / cpm of PMA/ionomycin stimulated MN cells). Each experiment consisted of quadruplicate samples and was performed at least twice.

Quantitative bioassay for TGFβ

9

The TGFβ concentration of serum-free MCF-7 CM was detected using a quantitative luciferase assay as published previously (41). This assay is based on the induction of a truncated TGFβ-responsive plasminogen activator inhibitor promoter driving luciferase expression and transfected into mink lung epithelial cells (gift from Dr. D. B. Rifkin, New York University, NY). The TGFβ concentrations of the samples were determined by comparison to a standard curve obtained with medium containing serial dilutions of recombinant $TGF\beta_1$ ($rTGF\beta_1$), $rTGF\beta_2$ and $rTGF\beta_3$ (R&D Systems, Minneapolis, MN). Luciferase activity was detected using an ML 10000 Luminometer (Dynatech, Chantilly, VA).

Quantitative bioassay for PGE_2 and elimination of PGE_2 by affinity binding

The expression of functional COX-1 on WM9 cells after transduction with AdCOX-1 and the concentration of PGE_2 in the CM from different breast cancer cell lines was confirmed by quantitative bioassay as follows. The amount of PGE_2 in the samples was tested by competitive enzyme-linked immunoassay (EIA) kit (Cayman) following the manufacturer's protocol. PGE_2 standards were reconstituted in serum-free RPMI medium and samples were tested undiluted. The percent standard bound/maximum bound ($\%B/B_0$) was calculated and plotted versus the PGE_2 concentration on a semi-log graph. The PGE_2 concentrations of the sample were determined by comparison to the standard curve. PGE_2 was eliminated from MCF-7 CM using a PGE_2 immunoaffinity column (Cayman). The column is designed for the specific purification of samples using a monoclonal anti-PGE_2 antibody covalently bound to sepharose with a loading capacity of 10 ng of PGE_2. The elimination of PGE_2 from MCF-7 CM was confirmed by EIA. Untreated MCF-7 CM and PGE_2 depleted MCF-7 CM were tested for their ability to inhibit the proliferation of stimulated MN cells. The depletion studies were performed twice.

Results

Adenoviral transfer of B7-1 to MCF-7 breast cancer cells.

We have shown previously that cultured human melanoma cells do not express B7-1 and do not co-stimulate proliferation of T cells in response to PHA (37). However, when transduced with AdhB7-1 these cells acquire the ability to co-stimulate T cells. Similar to human melanoma cells all nine cultured human breast cancer cells examined in this study do not have detectable levels of B7-1 on their cell surfaces (data not shown). To test whether B7-1 expression would confer co-stimulatory activity to breast cancer cells, we transduced MCF-7 cells with AdhB7-1. Flow cytometric analysis demonstrated that >90% of MCF-7 cells transduced with AdhB7-1 expressed the B7-1 protein on their cell surface (data not shown).

MCF-7 CM inhibits T cell proliferation.

We previously reported that many human melanoma cells transduced to express B7-1 stimulate proliferation of purified human T cells (37). To assess the co-stimulatory activity of B7-1 expressed by MCF-7 cells, we measured the proliferation of allogeneic T cells in co-culture with B7-1 expressing MCF-7 cells in comparison with B7-1 expressing WM9 melanoma cells. Purified human T cells were co-culture with untransduced, B7-1 transduced, or lacZ transduced tumor cells. B7-1 expressing WM9 cells induced at least a 53 fold increase in T cell proliferation (Table 1) over that achieved by untransduced WM9 cells. This effect was not due to the presence of adenovirus as seen by the low stimulation index (SI) of WM9/lacZ cells (Table 1). In contrast, there was no difference in T cell proliferation when untransduced MCF-7 cells or cells transduced with AdhB7-1 or AdlacZ were co-cultured with purified human T cells. In the absence of a co-stimulatory signal (e.g.. T cells alone) there is no proliferation (Table 1). To determine whether the lack of co-stimulation was due to secreted, soluble factors produced by MCF-7 cells, we added

11

MCF-7 conditioned medium (CM) to mononuclear (MN) cultures. MCF-7 CM blocked proliferation of MN preparations stimulated with either PHA (Fig. 1A) or PMA/ionomycin (Fig. 1B) indicating that MCF-7 CM exerts its effect independently of the stimulus used for T cell activation. Inhibition of lymphocyte proliferation was potent and dose-dependent with significant inhibition at 1:4,000 dilution (Fig. 1A, B). Co-culture of viable MCF-7 cells and lymphocytes separated by membranes impermeable to cells also led to inhibition of MN cell proliferation (data not shown).

Breast cancer cells produce soluble factors that suppress lymphocyte proliferation.

We sought to determine if the inhibitory effect of MCF-7 CM was present in other human breast cancer cell lines. Seven of eight additional breast cancer cell lines tested (BT-20, MCF-10, BT-474, MDA-MB 231, SUM52PE, SUM149PT and SUM190PT) also secreted soluble factors that were capable of inhibiting the proliferation of MN cells (Table 2). However, CM from SUM185 cells and the human breast epithelial HBL-100 cells did not inhibit lymphocyte proliferation (Table 2) under the same conditions. Thus, the production of soluble factors that inhibit lymphocyte proliferation is a common though not universal characteristic of human breast cancer cells.

Soluble TGFβ alone does not account for the inhibitory effect of MCF-7 CM on the proliferation of MN cells in response to PHA.

We next sought to determine whether MCF-7-derived TGFβ was responsible for the inhibitory effect of MCF-7 CM on T cell proliferation. Bioactive human recombinant TGFβ has been shown to inhibit proliferation of PHA stimulated T cells in a dose-dependent fashion (19), and MCF-7 cells reportedly secrete TGFβ (42). We confirmed that the MCF-7 cells used in the present study also secrete TGFβ at a rate of 1150 pg/1×10^6 cells in a 24 h period. To assess whether TGFβ in MCF-7 CM contributes to the inhibition

of lymphocyte proliferation, we evaluated the effect of MCF-7 CM on PHA-dependent MN cell proliferation in the presence of different concentrations of a neutralizing antibody that is reactive with all three known human TGFβ isoforms. The TGFβ antibody (up to 100 µg/ml, Fig. 2) was used in excess to block the biological effects of recombinant TGFβ at the concentration of TGFβ found in MCF-7 CM. That is, 100 µg/ml TGFβ antibody can neutralize 28 ng of total TGFβ. As determined by a TGFβ-responsive luciferase expression assay each sample in Fig. 2 contained 34.5 pg total TGFβ. However, the neutralizing TGFβ antibody (in amounts up to 162 fold excess over that needed for neutralization) had no effect on MCF-7 CM inhibition of MN proliferation (Fig. 2). These results effectively exclude TGFβ as the sole source of the inhibitory effect.

Indomethacin treatment of breast cancer cells restores lymphocyte proliferation.

COX is expressed in some human breast cancers (35, 36). To investigate whether the inhibition of COX activity restores lymphocyte poliferation, MCF-7 cells were treated with indomethacin (Cayman, Ann Arbor, MI) at a concentration of 100 µg/ml for 24 hours in RPMI medium containing 10% heat inactivated FBS. Indomethacin inhibits both COX-1 and COX-2 and reduced the PGE$_2$ production by MCF-7 cells to less than 30 pg/ml (the lower limit of detection in this assay, data not shown). Indomethacin pretreatment abrogated the inhibitory effect of MCF-7 CM on PMA/ionomycin stimulated proliferation of MN cells at a 1:4,000 dilution (Fig. 3A). However, the proliferation of MN cells was still inhibited when untreated MCF-7 CM or CM from indomethacin-treated MCF-7 cells that was more concentrated (1:40 dilution) was added to MN cells in the presence of mitogen. This is consistent with our observation that MCF-7 CM from indomethacin treated cells still contained residual amounts of PGE$_2$ detectable by liquid chromatograph-mass spectroscopy (LC-MS, data not shown). Indomethacin containing medium itself (not exposed to tumor cells) did not influence the proliferation of MN cells in response to

13

PMA/ionomycin (data not shown). We next performed T cell co-culture assays using MDA MB 231 cells that normally produced high amounts of PGE_2. We sought to test whether the treatment of B7-1 expressing MDA MB 231 cells with COX inhibitors reconstitutes T cell proliferation. Therefore, MDA MB 231 cells were transduced to express B7-1 and treated with indomethacin or NS398, a COX-2 specific inhibitor. As a control, MDA MB 231 cells and T cells were co-cultured in ethanol containing medium since ethanol served as a solvent for the COX inhibitors. B7-1 expressing MDA MB 231 cells produced 312 pg/ml PGE_2 and there were no detectable levels of PGE_2 after the treatment with either COX inhibitor at detectable levels (data not shown). Untreated or untransduced MDA MB 231 cells failed to stimulate allogeneic T cells in the absence of PHA (Fig. 3B). However, T cells were stimulated to proliferate when co-cultured with B7-1 expressing, indomethacin treated tumor cells in the presence of PHA. In contrast, there was no thymidine incorporation by T cells co-cultured with ethanol treated or NS398 treated B7-1 expressing MDA MB 231 cells or with unmodified MDA MB 231 cells in PHA containing medium (Fig. 3B). These results indicate that the inhibition of COX by indomethacin restores T cell proliferation although this effect was observed only in the presence of PHA.

PGE_2 production by breast carcinoma cells.

PGE_2, a known modulator of T cell proliferation (30, 31) has been shown to be produced by some breast carcinomas (16). We measured PGE_2 production by normal breast epithelium and by the breast cancer cell lines used in this study. We found that eight of ten cell lines produced PGE_2 at levels detectable by ELISA assay (Table 2). Interestingly, MCF-7 cells produced relatively low levels of PGE_2 (40 pg/ml) when compared to SUM190PT or SUM149PT cells which produce in excess of 1000 pg/ml PGE_2. With the exception of HBL-100, all of the cell lines that produced PGE_2 also inhibited the proliferation of PHA stimulated MN cells although there was no correlation between the

14

amount of secreted PGE_2 and the inhibitory capacity. In addition. BT-20 CM had a significant inhibitory effect although it did not produce detectable levels of PGE_2. Taken together, these results suggest that PGE_2 may play an important role in the inhibition of a proliferative response of lymphocytes, although this does not exclude the contribution of other factors to this process.

Breast cancer derived PGE_2 inhibits not only a mitogenic lymphocyte response but also B7-1 induced T cell proliferation.

As mentioned above. indomethacin did not completely eliminate PGE_2 and is expected to have reduced the levels of other prostaglandins in the CM. For example. $PGF_{2\alpha}$ is also present in similar amounts in MCF-7 CM. although unlike PGE_2, addition of $PGF_{2\alpha}$ does not inhibit MN cell proliferation (data not shown). Therefore. we sought to remove PGE_2 more effectively from MCF-7 CM and determined whether PGE_2 alone was responsible for the inhibitory effect. We used an immunoaffinity column that specifically eliminates up to 10 ng PGE_2 (62 fold more than the loaded amount in the CM). MCF-7 CM passed through this immunoaffinity column completely lost its inhibitory effect on MN proliferation at all dilutions tested (Fig. 4A). These results indicate that breast cancer derived PGE_2 blocks mitogenic lymphocyte responses.

The effect of PGE_2 on mitogenic lymphocyte responses has been reported before (29, 10), whereas the effect of PGE_2 on B7-1 dependent T cell proliferation has not been studied in great detail. Therefore. we sought to investigate the role of PGE_2 on immune responses in a more defined system where there is no endogenous source of PGE_2 and B7-1. When MCF-7 CM was added to T cells co-cultured with B7-1 expressing melanoma cells the proliferation of T cells was completely inhibited (Fig. 4B. diamond). As shown in Fig. 4B. the depletion of PGE_2 from MCF-7 CM abrogated the proliferation of T cells in the presence of PHA (circle). whereas the addition of exogenous PGE_2 to MCF-7 CM blocked

thymidine uptake of stimulated T cells (triangle). These data indicate that PGE_2 plays an important role in the inhibition of B7-1 induced T cell proliferation.

Melanoma cells transduced to produce PGE_2 block B7-1 dependent T cell proliferation

To support the hypothesis that tumor cells block B7-1 dependent T cell mediated immune responses through the production of PGE_2, we generated PGE_2 producing and B7-1 positive melanoma WM9 cells by transduction with AdCOX-1 and AdhB7-1. Proliferation assays using T cells co-cultured with WM9 cells modified to express B7-1 and to produce PGE_2 were performed. T cells were stimulated to proliferate by the co-culture with B7-1 expressing WM9 cells in the absence or presence of PHA (Fig 5). However, the proliferation of T cells was inhibited when incubated with WM9 cells transduced to express B7-1 and COX-1. There was a four fold increase of PGE_2 in the five days supernatant of T cells co-cultured with B7-1, COX-1 expressing WM9 cells (538 pg/ml) as compared to the supernatant from T cells co-cultured with B7-1 expressing WM9 cells. The allogeneic T cell response of B7-1 expressing WM9 cells was completely restored by the co-culture of T cells with WM9 cells transduced with a mixture of AdhB7-1 and AdBgl2. These results clearly indicate that the expression of COX-1 and the resulting production of PGE_2 by WM9 cells inhibits B7-1 induced T cell proliferation.

antibody used were large excess of that needed to neutralize the effects of TGFβ in a sensitive TGFβ-responsive luciferase reporter assay.

In contrast, PGE$_2$ was shown to be a more important contributor to the T cell inhibitory effect of breast cancer CM. Several lines of evidence support this conclusion. Indomethacin treatment of MCF-7 cells reduced PGE$_2$ production and partially alleviated inhibition of MN cell proliferation. Indomethacin did not completely remove the inhibitory effect which is consistent with the presence of residual PGE$_2$ that could be detected by LC-MS. In the presence of PHA B7-1 expressing MDA MB 231 cells stimulated T cells to proliferate when the modified tumor cells were treated with indomethacin prior co-culture. More conclusive evidence for the involvement of PGE$_2$ in T cell growth inhibition was obtained when we removed PGE$_2$ from CM using an affinity column that specifically binds PGE$_2$. The selective elimination of PGE$_2$ from MCF-7 CM removed its MN cell growth inhibitory activity completely. Thus, inhibition of MN cell proliferation is mediated, at least in large part, by PGE$_2$ produced by MCF-7 cells. Furthermore, PGE$_2$ was detected in CM from additional breast cancer cell lines that also inhibited MN cell proliferation. The amount of PGE$_2$ production did not linearly correlate with inhibition of the proliferation of stimulated MN cells (Table 2). CM from the normal human breast epithelial cell line HBL-100 produced significant levels of PGE$_2$ (more than MCF-7) but did significantly suppress MN cell proliferation. Similarly, the CM from two cell lines (SUM149PT and SUM190PT) that produced the most PGE$_2$ showed only moderate inhibition of MN cell proliferation (Table 2). Although the minimal amount of PGE$_2$ needed to inhibit T cell proliferation has not been established, it appears likely that other factors in the breast cancer CM likely contribute to the effect. In support of this is the observation that BT-20 cells did not produce significant amounts of PGE$_2$ but inhibited PHA-dependent proliferation of MN cells by 41% (Table 2). This suggests that other tumor derived factors may induce immunosuppression as previously reported (45, 46). Nevertheless, seven of nine breast

cancer cell lines produced PGE_2, and all inhibited the proliferative response of MN cells to PHA. This, accompanied by the PGE_2 depletion data, suggests that PGE_2 contributes to tumor derived immunosuppression, although it is likely not the sole factor. Several reports support a role of PGE_2 in diseases with impaired immunological responses including bone marrow and stem cell transplantation (Cayeux SJ, Dorken B, 1993 Bone Marrow Transplant 12) and AIDS (Mastino A, 1993 Cell Immunol 152; Dellemare FG 1995 AIDS 9).

The mechanism(s) by which PGE_2 affects MN or T cell proliferation are as yet fully understood. Intracellularly, PGE_2 was found to attenuate p59fyn phosphorylation and its kinase activity, thus suppressing T cell proliferation during burn and sepsis (Choudhry MA 1998 J Immun 160, 2, p929). Both suppression of lymphokine production (33, 34) and downregulation of MHC class II expression on antigen presenting cells (30, 31) by PGE_2 have been demonstrated. PGE_2 inhibits IL-2 production and enhances IL-4 production by T cell thus shifting a T helper cell 1 response towards a T helper cell 2 response (Katamura K 1995, J Immunol 155). Recently, small cell lung cancer-derived PGE_2 was found to up-regulate IL-10 production by lymphocytes and to down-regulate IL-12 production by macrophages (47), whereas in our study pure T cell cultures were used to investigate the effect of breast cancer derived PGE_2. Furthermore, the expression of CD40L on human memory T cells was blocked by PGE_2 (Splawski JB, J Immun 96). These studies suggest that PGE_2-dependent inhibition of T cell-mediated anti-tumor responses simultaneously affects antigen presentation and shifts the local cytokine milieu to a state unfavorable to an effective immune response (47).

The significance of tumor-derived PGE_2 to inhibition of cells of the immune system, e.g., natural killer cells is highlighted by recent reports demonstrating therapeutic efficacy in the prevention and treatment of breast cancer by non-steroidal anti-inflammatory drugs

19

(NSAID) including indomethacin itself (27). Importantly, indomethacin reduces PGE_2 production by inhibiting the enzymatic activity of the COX-1 and COX-2 which are rate-limiting enzymes in the PGE_2 biosynthetic pathway. Indomethacin has also demonstrated efficacy in the treatment of mouse mammary carcinoma cells. Mice injected with mammary adenocarcinoma C3-L5 cells received long-term indomethacin therapy on day 15 followed by two rounds of IL-2 administration for five days. Regression of primary tumors, reduction of lung metastases and prolonged survival were observed in the group receiving the combination therapy as opposed to the group receiving IL-2 treatment alone. Furthermore, the long-term intake of indomethacin in combination with IL-2 was shown to activate tumoricidal lymphocytes in situ (27). Here we show that a combination strategy of B7-1 transfer onto breast cancer cells along with indomethacin treatment activated the proliferation of T cells in vitro.

B7-1 immunotherapy has been reported to be effective in immunogenic tumors (Chen L J Ex ed 94). Tumor cells are generally termed "immunogenic" when they express antigens that result in the rejection of tumor cells by syngeneic animals previously immunized with the irradiated parental tumor cells (Chen L J Ex Med 94). Accordingly, non-immunogenic tumors are rejected when similarly tested. However, there is no consensus about the parameters that render tumor cells more or less immunogenic. Breast cancer cells are known as non-immunogenic tumors and thus escape immune surveillance. Here, B7-1 immunotherapy was not successful in vitro in stimulating T cell proliferation. The majority of non-immunogenic breast cancer cell lines produce PGE_2. We were able to show that the production of PGE_2 specifically inhibited B7-1 induced T cell proliferation. First, B7-1 expressing MDA MB 231 cells did not secrete PGE_2 at detectable levels after indoemthacin treatment and stimulated T cells to proliferate when PHA was present. Second, the elimination of PGE_2 from MCF-7 CM restored the response of T cells to B7-1 expressing melanoma cells only in the presence of PHA indicating that the inhibition by PGE_2 can only

be overcome when a strong signal through the T cell receptor is provided. Finally, in the melanoma system the sole modification of co-expression of COX-1 on B7-1 expressing WM9 cells was sufficient to inhibit T cell proliferation. These results underline the potential of a combined modification of breast cancer cells consisting of B7-1expression and reduction of PGE_2 in order to induce T cell proliferation.

In summary, this study provides evidence that PGE_2 derived from human breast cancer cells can contribute to inhibition of cellular immunity in vitro. Since levels of PGE_2 are elevated in at least some breast cancers (48, 49), the production of PGE_2 may contribute to the impaired anti-tumor immune response. Reversal of tumor-induced immunosuppression offers a potential approach to cancer therapy and may be particularly useful in combination with immunotherapy against breast cancer.

Acknowledgement

References

1. Boon, T., Cerottini, J. C., Van den Eynde. B.. van der Bruggen, P., and Van PeL A. Tumor antigens recognized by T lymphocytes. Ann. Rev. Immunol. *12:* 337-365. 1994.

2. Schwartz, R. H. Costimulation of T lymphocytes: the role of CD28, CTLA-4. and B7/BB1 in interleukin-2 production and immunotherapy, Cell, *71:* 1065-1068, 1992.

3. Daniel, P. T., Kroidl, A., Cayeux, S.. Bargou. R.. Blankenstein, T., and Dorken. B. Costimulatory signals through B7.1/CD28 prevent T cell apoptosis during target cell lysis, J. Immunol., *159:* 3808-3815, 1997.

4. Harding, F. A., McArthur, J. G., Gross. J. A.. Raulet, D. H., and Allison, J. P. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones., Nature, *356:* 607-609. 1992.

5. Chen, L., Ashe, S., Brady, W. A., Hellstrom. L. Hellstrom, K. E., Ledbetter, J. E.. McGowan, P., and Linsley, P. S. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4., Cell, *71:* 1093-1102, 1992.

6. Townsend, S. E. and Allison, J. P. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells.. Science. *259:* 368-370, 1993.

7. Allison, J. P. CD28-B7 interactions in T-cell activation, Curr. Opin. Immunol. *6:* 414-419, 1994.

8. Coughlin, C. M., Wysocka, M., Kurzawa. H. L., Lee, W. M., Trinchieri, G., and Eck, S. L. B7-1 and interleukin 12 synergistically induce effective antitumor immunity, Cancer Res., *55:* 4980-4987. 1995.

9. Li, Y., Hellstrom, K. E., Newby, S. A.. and Chen. L. Costimulation by CD48 and B7-1 induces immunity against poorly immunogenic tumors, J. Exp. Med.. *183:* 639-644, 1996.

10. Baskar, S., Ostrand-Rosenberg, S., Nabavi, N., Nadler, L. M., Freeman, G. J., and Glimcher, L. H. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules, PROC. NATL. ACAD. SCI.., *90:* 5687-5690, 1993.

11. Martin-Fontecha, A., Cavallo. F., Bellone, M., Heltai, S., Iezzi, G., Tornaghi, P., Nabavi, N., Forni, G., Dellabona. P., and Casorati, G. Heterogeneous effects of B7-1 and B7-2 in the induction of both protective and therapeutic anti-tumor immunity against different mouse tumors. Eur. J. Immunol., *26:* 1851-1859, 1996.

12. Matulonis, U., Dosiou, C., Freeman, G., Lamont, C., Mauch, P., Nadler, L. M., and Griffin, J. D. B7-1 is superior to B7-2 costimulation in the induction and maintenance of T cell-mediated antileukemia immunity. Further evidence that B7-1 and B7-2 are functionally distinct. J. Immunol., *156:* 1126-1131, 1996.

13. Chen, L., McGowan. P., Ashe. S., Johnston, J., Li, Y., Hellstrom, I., and Hellstrom, K. E. Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. J. Exp. Med., *179:* 523-532, 1994.

14. McCune, B. K., Mullin. B. R., Flanders, K. C., Jaffurs, W. J., Mullen, L. T., and Sporn, M. B. Localization of transforming growth factor-beta isotypes in lesions of the human breast, Human Path., *23:* 13-20. 1992.

15. Venetsanakos, E., Beckman. I., Bradley, J., and Skinner, J. M. High incidence of interleukin 10 mRNA but not interleukin 2 mRNA detected in human breast tumours, Brit. J. Cancer, *75:* 1826-1830. 1997.

16. Karmali, R. A., Welt. S., Thaler. H. T., and Lefevre, F. Prostaglandins in breast cancer: relationship to disease stage and hormone status, Brit. J. Cancer, *48:* 689-696, 1983.

17. Massague, J. The transforming growth factor-beta family, Ann. Rev. Cell Biol., *6:* 597-641, 1990.

18. Torre-Amione, G., Beauchamp, R. D., Koeppen, H., Park, B. H., Schreiber, H., Moses, H. L., and Rowley, D. A. A highly immunogenic tumor transfected with a murine transforming growth factor type beta 1 cDNA escapes immune surveillance, PNAS., *87:* 1486-1490, 1990.

19. Arteaga, C. L., Hurd, S. D., Winnier, A. R., Johnson, M. D., Fendly, B. M., and Forbes, J. T. Anti-transforming growth factor (TGF)-beta antibodies inhibit breast cancer cell tumorigenicity and increase mouse spleen natural killer cell activity. Implications for a possible role of tumor cell/host TGF-beta interactions in human breast cancer progression, J. Clin. Invest., *92:* 2569-2576, 1993.

20. Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M. B., and Fauci, A. S. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth, J. Exp. Med., *163:* 1037-1050, 1986.

21. Moore, K. W., O'Garra, A., de Waal Malefyt, R., Vieira, P., and Mosmann, T. R. Interleukin-10, Ann. Rev. Immunol., *11:* 165-190, 1993.

22. Ding, L., Linsley, P. S., Huang, L. Y., Germain, R. N., and Shevach, E. M. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression, J. Immunol., *151:* 1224-1234, 1993.

23. Fernandez-Botran, R., Sanders, V. M., Mosmann, T. R., and Vitetta, E. S. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells, J. Exp. Med., *168:* 543-558, 1988.

24. Fiorentino, D. F., Bond, M. W., and Mosmann, T. R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones, J. Exp. Med., *170:* 2081-2095, 1989.

25. Hsu, D. H., Moore, K. W., and Spits, H. Differential effects of IL-4 and IL-10 on IL-2-induced IFN-gamma synthesis and lymphokine-activated killer activity, Internatl. Immunol., *4:* 563-569, 1992.

25

26. Brunda, M. J., Herberman, R. B., and Holden, H. T. Inhibition of murine natural killer cell activity by prostaglandins, J. Immunol., *124:* 2682-2687, 1980.

27. Lala, P. K., Parhar, R. S., and Singh, P. Indomethacin therapy abrogates the prostaglandin-mediated suppression of natural killer activity in tumor-bearing mice and prevents tumor metastasis, Cell. Immunol., *99:* 108-118, 1986.

28. Skibinski, G., Kelly, R., Harrison, C., McMillan, L., and James, K. Relative immunosuppressive activity of human seminal prostaglandins., J. Reprod. Immunol., *22:* 185-195, 1992.

29. Miao, D., Skibinski, G., and James, K. The effects of human seminal plasma and PGE2 on mitogen induced proliferation and cytokine production of human splenic lymphocytes and peripheral blood mononuclear cells, J. Reprod. Immunol., *30:* 97-114, 1996.

30. Valitutti, S., Castellino, F., Aiello, F. B., Ricci, R., Patrignani, P., and Musiani, P. The role of arachidonic acid metabolite PGE_2 on T cell proliferative response, J. Clin. & Lab. Immunol., *29:* 167-173, 1989.

31. Laning, J. C., Isaacs, C. M., and Hardin-Young, J. Normal human keratinocytes inhibit the proliferation of unprimed T cells by TGFbeta and PGE2, but not IL-10, Cell. Immunol., *175:* 16-24, 1997.

32. Arvind, P., Papavassiliou, E. D., Tsioulias, G. J., Qiao, L., Lovelace, C. I., Duceman, B., and Rigas, B. Prostaglandin E2 down-regulates the expression of HLA-DR antigen in human colon adenocarcinoma cell lines, Biochemistry, *34:* 5604-5609, 1995.

33. Murray, J. L., Dowd, J., and Hersh, E. M. In vitro inhibition of interleukin-2 production by peripheral blood lymphocytes from stage III melanoma patients by prostaglandin E2: enhancement of lymphocyte proliferation by exogenous interleukin-2 plus indomethacin, J. Biol. Response Modifiers, *5:* 12-19, 1986.

34. Vercammen, C. and Ceuppens, J. L. Prostaglandin E_2 inhibits human T-cell proliferation after crosslinking of the CD3-Ti complex by directly affecting T cells at an early step of the activation process, Cellular Immunology. *104:* 24-36, 1987.

35. Liu, X. H. and Rose, D. P. Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines, Cancer Research. *56:* 5125-7, 1996.

36. Hwang, D., Scollard, D., Byrne, J., and Levine, E. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer, Journal of the National Cancer Institute. *90:* 455-460, 1998.

37. Boxhorn, H. K. E., Smith, J. G., Chang, Y., Guerry, D., Lee, W. M. F., Rodeck, U., Turka, L. A., and Eck, S. L. Adenoviral transduction of melanoma cells with B7-1: anti-tumor immunity and immunosuppressive factors, Cancer Immunology Immunotherapy in press, 1998.

38. Ethier, S. P., Mahacek, M. L., Gullick, W. J., Frank, T. S., and Weber, B. L. Differential isolation of normal luminal mammary epithelial cells and brest cancer cells from primary and metaststic sites using selctive media., Cancer Res. *53:* 627-635, 1993.

39. Engelhardt, J. F., Yang, Y., Stratford-Perricaudet, L. D., Allen, E. D., Kozarsky, K., Perricaudet, M., Yankaskas, J. R., and Wilson, J. M. Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses, Nature Genetics. *4:* 27-34, 1993.

40. Roberts, A. B. and Sporn, M. B. The transforming-growth-βs., p. 419-472. Heidelberg: Springer-Verlag, 1990.

41. Bellone, G., Silvestri, S., Artusio, E., Tibaudi, D., Turletti, A., Geuna, M., Giachino, C., Valente, G., Emanuelli, G., and Rodeck, U. Growth stimulation of colorectal carcinoma cells via the c-kit receptor is inhibited by TGF-beta 1, Journal of Cellular Physiology. *172:* 1-11, 1997.

42. Arteaga, C. L., Carty-Dugger, T., Moses, H. L., Hurd, S. D., and Pietenpol, J. A. Transforming growth factor beta 1 can induce estrogen-independent tumorigenicity of human breast cancer cells in athymic mice, Cell Growth & Differentiation. *4:* 193-201, 1993.

43. Kruisbeek, A. In vitro assays for mouse lymphocyte function, Vol. 1, p. 3.12.1-3.12.2. New York: John Wiley & Sons. 1996.

44. Truneh, A., Albert, F., Golstein, P., and Schmitt-Verhulst, A. M. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester, Nature. *313:* 318-20. 1985.

45. Strasnick, B., Lagos, N., Lichtenstein, A., and Mickel, R. A. First place--Resident Clinical Science Award 1990. Suppression of lymphokine-activated killer cell cytotoxicity by a soluble factor produced by squamous tumors of the head and neck, Otolaryngology - Head & Neck Surgery. *103:* 537-49, 1990.

46. O'Mahoney, A., O'Sillivan, G., O'Connell, J., Cotter, T., and Collins, J. An immune suppressive factor derived from esophageal squamous cell carcinoma induces apoptosis in normal and transformed cells of lymphoid lineage, Journal of Immunology. *151:* 4847-4856. 1993.

47. Huang, M., Stolina, M., Sharma, S., Mao, J., Zhu, L., Miller, P., Wollman, J., Herschman, H., and Dubinett, S. M. Non-small lung cancer cyclooxygenase-2-dependent regulation of cytokine balance in lymphocytes and macrophages: up-regulation od Interleukin 10 and down-regulation of interleukin 12 production, Cancer Research. *58:* 1208-1216. 1998.

48. Watson, J. and Chuah, S. Y. Technique for the primary culture of human breast cancer cells and measurement of their prostaglandin secretion, Clinical Science. *83:* 347-52. 1992.

28

49. Yang, C. Y. and Meng, C. L. Regulation of PG synthase by EGF and PDGF in human oral, breast, stomach, and fibrosarcoma cancer cell lines, Journal of Dental Research. *73:* 1407-15, 1994.

Table 1. [^{3}H]Thymidine uptake (cpm) and relative proliferation of human T cells cultured with B7-1 expressing MCF-7 cells or with B7-1 expressing WM-9 cells. Purified T cells were co-cultured with untransduced, AdB7-1 transduced or AdlacZ transduced tumor cells. Their proliferation is expressed relative that of T cells in the absence of tumor cells as indicated by SI. [^{3}H]Thymidine incorporation (mean cpm) and standard deviation (sd) were obtained from quadruplicates. B7-1 expressing MCF-7 cells failed to stimulate T cell proliferation.

Table 2. PGE$_2$ levels and inhibition of PHA stimulated MN cell proliferation by CM from a normal human breast epithelial cell line (HBL-100) and human breast carcinoma cell lines (all others).

Fig. 1. [^{3}H]Thymidine uptake (cpm) of MN cells stimulated with (A) PHA or (B) PMA/ionomycin after the addition of MCF-7 CM. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. MCF-7 CM inhibited the proliferation of stimulated MN cells.

Fig. 2. The effect of MCF-7 CM on the proliferation (thymidine uptake in cpm) of PHA stimulated MN cells after incubation with a pan-specific TGFβ neutralizing antibody (100 µg/ml to 2.5 ng/ml). Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. TGFβ did not account for the inhibitory effect of the MCF-7 CM.

Fig. 3. Indomethacin treatment of breast cancer cells restores the proliferation of lymphocytes. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. (A) [^{3}H]Thymidine incorporation by MN cells stimulated with PMA/ionomycin in the presence of absence of CM from MCF-7 cells treated with
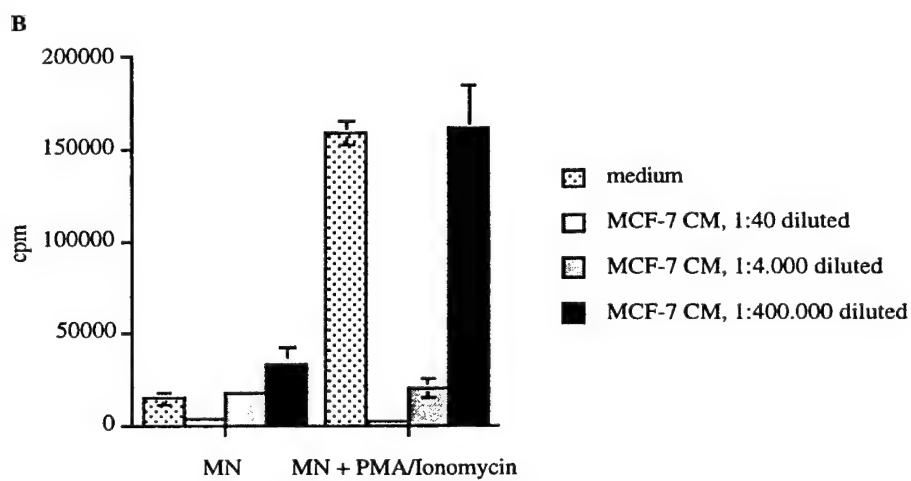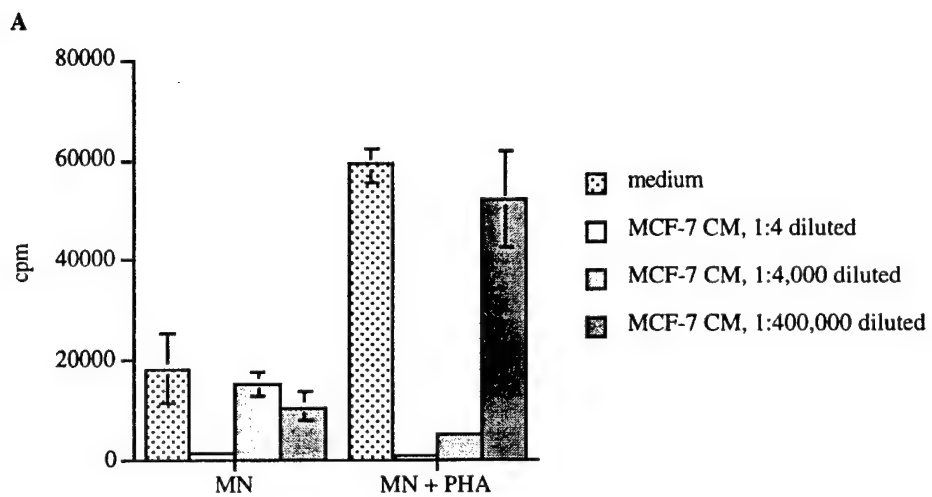
indomethacin. Indomethacin treatment of MCF-7 cells partially alleviated the immunosuppressive effect of MCF-7 CM. (B) [^{3}H]Thymidine incorporation by T cells co-cultured with B7-1 expressing MDA MB 231 cells after the treatment with indomethacin, NS 398 or ethanol. In the presence of PHA indomethacin treatment of B7-1 expressing MDA MB 231 cells abrogated T cell proliferation.
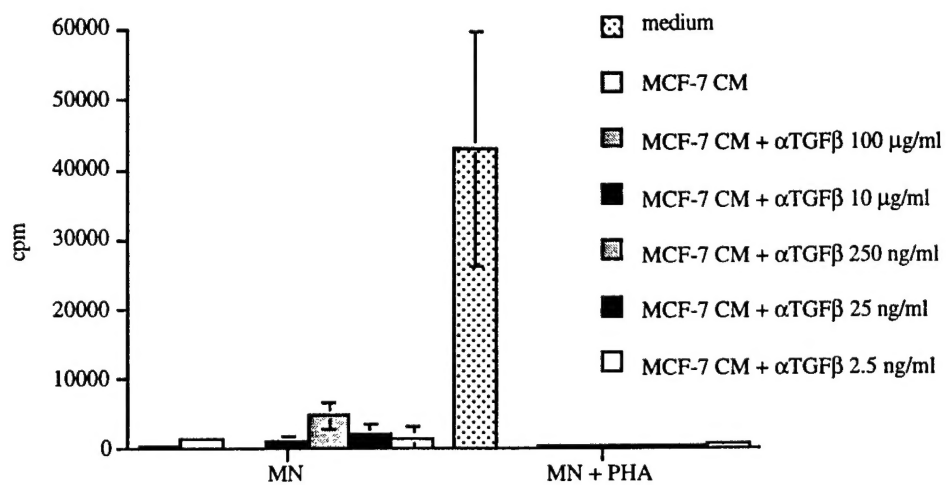
Fig. 4. Breast cancer derived inhibits a mitogenic response of lymphocytes (A) and B7-1 induced T cell proliferation (B). Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. (A) [^{3}H]thymidine incorporation by MN cells (square), by MN cells stimulated with PMA/ionomycin (diamond), by PMA/ionomycin stimulated MN cells incubated with MCF-7 CM (circle) and by PMA/ionomycin stimulated MN cells incubated with PGE$_2$-depleted MCF-7 CM (triangle). MCF-7 CM inhibited the mitogenic response of MN cells. The inhibitory effect of MCF-7 CM was completely alleviated when PGE$_2$ was eliminated via PGE$_2$ affinity column. (B) [^{3}H]thymidine incorporation by T cells co-cultured with B7-1 expressing WM9 cells after the addition of MCF-7 CM (diamond), PGE$_2$ depleted MCF-7 CM (circle) and MCF-7 CM previously depleted of PGE$_2$ with exogenous PGE$_2$ (triangle). In the presence of PHA the proliferation of T cells co-cultured with B7-1 expressing WM9 cells and PHA was inhibited in the presence of MCF-7 CM or in the presence of MCF-7 CM that had been depleted from PGE$_2$ and substituted with exogenous PGE$_2$. The proliferation of T cells in response to B7-1 expressing WM9 cells and PHA was restored when PGE$_2$ depleted MCF-7 CM was added.

Fig. 5. [^{3}H]Thymidine incorporation by T cells co-cultured with WM9 cells co-expressing COX-1 and B7-1. Data were obtained as the mean of quadruplicates and error bars represent the standard deviation. The proliferation of T cells in response to B7-1 expressing WM9 cells was inhibited by the co-expression of COX-1 on the tumor cells.
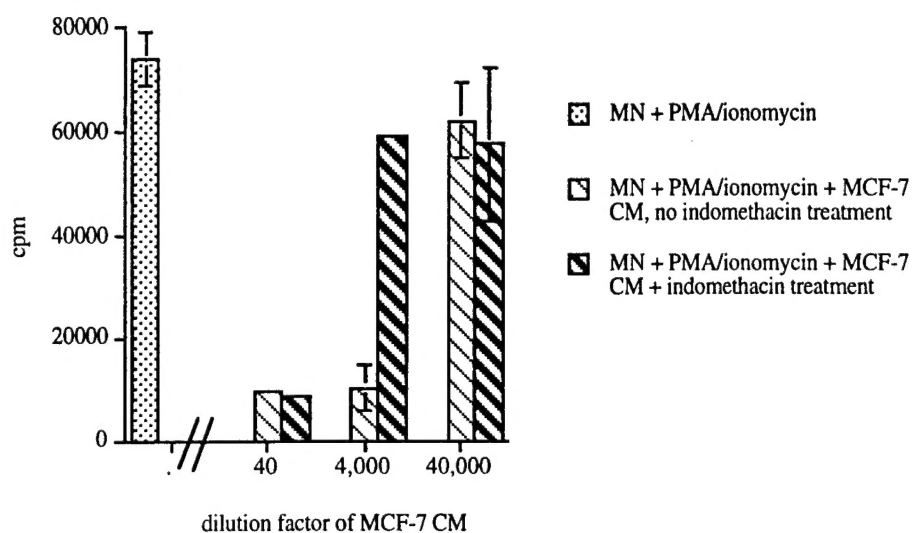
31

Tumor cells	T cells	cpm	sd	SI
MCF-7	-	39	7	/
	+	55	8	0.3
MCF-7/B7-1	-	48	6	/
	+	551	169	0.9
MCF-7/lacZ	-	37	7	/
	+	43	6	0.1
WM9	-	25	4	/
	+	1287	109	24
WM9/B7-1	-	32	6	/
	+	68374	11736	1340
WM9/lacZ	-	291	55	/
	+	300	236	0.2
	T cells alone	51	6	/

human cell line	% inhibition of PHA stimulated MN cells	PGE_2 production (pg/ml)
HBL-100* (breast epithelial)	5%	100
BT-20	41%	0
MCF-10	99%	300
MCF-7	97%	40
BT-474	96%	40
MDA-MB 231	90%	260
SUM52PE	66%	30
SUM149PT	56%	>1000
SUM185PE	4%	0
SUM190PT	51%	>1000

33

A

Legend:
- medium
- MCF-7 CM, 1:4 diluted
- MCF-7 CM, 1:4,000 diluted
- MCF-7 CM, 1:400,000 diluted

(X-axis: MN, MN + PHA; Y-axis: cpm, 0 to 80000)

B

Legend:
- medium
- MCF-7 CM, 1:40 diluted
- MCF-7 CM, 1:4.000 diluted
- MCF-7 CM, 1:400.000 diluted

(X-axis: MN, MN + PMA/Ionomycin; Y-axis: cpm, 0 to 200000)

34

A

dilution factor of MCF-7 CM

Legend:
- MN + PMA/ionomycin
- MN + PMA/ionomycin + MCF-7 CM, no indomethacin treatment
- MN + PMA/ionomycin + MCF-7 CM + indomethacin treatment

B

Legend:
- MDA MB 213
- MDA MB 213 AdhB7
- MDA MB 213 AdhB7 + NS 398
- MDA MB 213 AdhB7 + Indomethacin
- MDA MB 213 AdhB7 + EtOH
- MDA MB 213 AdBgl2

X-axis labels: cells alone, + Tcells, + Tcells + PHA

A

dilution factor of MCF-7 CM

B